A Substrate-induced Biotin Binding Pocket in the Carboxyltransferase Domain of Pyruvate Carboxylase

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A Substrate-induced Biotin Binding Pocket in the Carboxyltransferase Domain of Pyruvate Carboxylase*

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Abstract

Biotin-dependent enzymes catalyze carboxyl transfer reactions by efficiently coordinating multiple reactions between spatially distinct active sites. Pyruvate carboxylase (PC), a multifunctional biotin-dependent enzyme, catalyzes the bicarbonate- and MgATP-dependent carboxylation of pyruvate to oxaloacetate, an important anaplerotic reaction in mammalian tissues. To complete the overall reaction, the tethered biotin prosthetic group must first gain access to the biotin carboxylase domain and become carboxylated and then translocate to the carboxyltransferase domain, where the carboxyl group is transferred from biotin to pyruvate. Here, we report structural and kinetic evidence for the formation of a substrate-induced biotin binding pocket in the carboxyltransferase domain of PC from *Rhizobium etli*. Structures of the carboxyltransferase domain reveal that *R. etli* PC
occupies a symmetrical conformation in the absence of the biotin carboxylase domain and that the carboxyltransferase domain active site is conformationally rearranged upon pyruvate binding. This conformational change is stabilized by the interaction of the conserved residues Asp$^{590}$ and Tyr$^{628}$ and results in the formation of the biotin binding pocket. Site-directed mutations at these residues reduce the rate of biotin-dependent reactions but have no effect on the rate of biotin-independent oxaloacetate decarboxylation. Given the conservation with carboxyltransferase domains in oxaloacetate decarboxylase and transcarboxylase, the structure-based mechanism described for PC may be applicable to the larger family of biotin-dependent enzymes.

**Keywords:** Biotin, Decarboxylase, Enzyme Catalysis, Enzyme Kinetics, Enzyme Mechanisms, Enzyme Structure, Pyruvate Carboxylase

**Introduction**

Biotin-dependent enzymes catalyze carboxyl transfer reactions on a range of metabolically important substrates and are widely distributed across all three domains of life (recently reviewed in Refs. 1 and 2). This family of enzymes includes acetyl-CoA carboxylase, propionyl-CoA carboxylase, the oxaloacetate decarboxylase complex, transcarboxylase, and pyruvate carboxylase, all of which act in central metabolism and serve to maintain cellular homeostasis. Pyruvate carboxylase (PC$^3$; EC 6.4.1.1) catalyzes the bicarbonate- and MgATP-dependent carboxylation of pyruvate to oxaloacetate. This important anaplerotic reaction serves to replenish oxaloacetate in the tricarboxylic acid cycle, where intermediates are routinely funneled to other metabolic pathways, such as gluconeogenesis in the liver (3), fatty acid biosynthesis in adipose tissue (4), neurotransmitter biosynthesis in astrocytes (5, 6), and glucose-mediated insulin secretion in pancreatic islets (7). Type II diabetes (8, 9), tumor cell proliferation (10–12), and bacterial virulence (13, 14) are all associated with aberrant PC expression levels and enzyme activity.

In all eukaryotes and most prokaryotes, PC is a homotetramer. Each monomer is 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. The N-terminal BC domain initiates the overall reaction by catalyzing the MgATP- and bicarbonate-dependent carboxylation of biotin. The carboxylated biotin cofactor, which is covalently tethered via an amide linkage to the side chain of a strictly conserved lysine in the BCCP domain, then physically translocates ~60 Å across the subunit cleft to a neighboring CT domain active site where the carboxyl moiety is transferred to the acceptor substrate, pyruvate (15–18).

The chemical mechanisms for the individual BC (19) and CT (20) domain reactions have been well characterized. For example, in the CT domain of *Rhizobium etli* PC (RePC), Thr882 acts to shuttle a proton from the biotin enolate to pyruvate (20), whereas Arg548, Gln552, and Arg621 serve to stabilize the pyruvate enolate (16, 21, 22) (Scheme 1). Furthermore, there is ample kinetic evidence suggesting that the spatially distinct reactions in the BC and CT domains are remarkably well coordinated (23). However, the molecular basis for this coordination remains largely unknown. Recently, we reported structures of RePC suggesting that BCCP-biotin is precluded from the BC domain active site following carboxylation (18). This protects against the abortive decarboxylation of carboxybiont in the BC domain that would result in an energetically wasteful decoupling of ATP hydrolysis from pyruvate carboxylation (19).
SCHEME 1.
The proposed carboxyltransferase reaction mechanism in *R. etli* PC.

Although a mechanism to protect against abortive decarboxylation has been proposed for the BC domain, it is not yet clear whether a related mechanism exists in the CT domain. Interestingly, PC-carboxybiotin is quite stable in the absence of substrates, but several competitive inhibitors of pyruvate are sufficient to invoke the translocation and decarboxylation of *N*-[^14]C]carboxybiotin in the CT domain (24). These experiments imply that 1) carboxybiotin can rapidly decarboxylate in the CT domain, even in the absence of the *bona fide* acceptor substrate, and 2) a CT domain ligand is required to facilitate biotin access to the CT domain active site. It is clear from structural studies (16) and kinetic isotope effects (25) that PC catalyzes biotin decarboxylation in the CT domain without direct transfer of the carboxyl group from biotin to pyruvate.
However, existing structural models are unable to explain how the CT domain ligand facilitates biotin access and decarboxylation. This difficulty stems, in large part, from an absence of directly comparable structures of PC.

Here we report a series of directly comparable structures and accompanying kinetic studies that reveal a substrate-induced biotin binding pocket in the CT domain of RePC. The active site of the CT domain undergoes a conformational change upon pyruvate binding. This conformational rearrangement, which is stabilized by the interaction of conserved Asp\textsuperscript{590} and Tyr\textsuperscript{628} in RePC, results in formation of the biotin binding pocket. Due to the conserved nature of both Asp\textsuperscript{590} and Tyr\textsuperscript{628} and similar active site architecture in homologous biotin-dependent enzymes, we hypothesize that the structural mechanism described here for PC is applicable to the broader family of biotin-dependent enzymes.

**Experimental Procedures**

**General**

Isopropyl 1-thio-β-D-galactopyranoside, D-biotin, ampicillin, kanamycin, chloramphenicol, DL-dithiothreitol (DTT), epoxysuccinyl-L-leucylamido(4-guanido) butane, and pepstatin A were purchased from Research Products International Corp. (Mount Prospect, IL). Malate dehydrogenase and lactate dehydrogenase were purchased from Roche Applied Science. Acetyl-CoA was purchased from Crystal Chem, Inc. (Downers Grove, IL). All other materials were purchased from Sigma-Aldrich. RePC and ΔBC RePC were previously subcloned into a modified pET\textsuperscript{−}17b and pET\textsuperscript{−}28a vector, respectively, for expression in λ(DE3) lysogenized *Escherichia coli* (15, 18).

**Site-directed Mutagenesis and Domain Truncation Mutations**

The Y628F, Y628A, and D590A mutations were generated using the QuikChange site-directed mutagenesis protocol (Agilent Technologies, Santa Clara, CA) and were verified by complete
sequencing of the gene by Functional Biosciences, Inc. (Madison, WI). For the BCCP domain truncation, it was determined that Ala$^{1067}$ represents the N-terminal end of the BCCP domain based on the x-ray crystal structure of RePC (PDB code 2QF7). The BC and BCCP domain deletion construct (ΔBCΔBCCP RePC) was therefore generated via the QuikChange protocol by incorporating two stop codons after the codon for Ala$^{1067}$ in an expression vector encoding the BC domain truncation of RePC (18). The resulting ΔBCΔBCCP RePC construct retains the complete allosteric and carboxyltransferase domains of RePC. Identical mutagenic oligonucleotide primers were used to generate the Tyr$^{628}$ and Asp$^{590}$ mutations in both full-length RePC and ΔBCΔBCCP RePC. Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA).

RePC and ΔBCΔBCCP RePC Protein Production and Purification

RePC and all mutant forms of the full-length enzyme were co-expressed with the pCY216 vector encoding *E. coli* biotin protein ligase (BirA) in *E. coli* BL21Star(DE3) cells from a modified pET-17b vector as described previously (18). Cells were cultured in M9 minimal medium containing 200 mg/liter ampicillin and 30 mg/liter chloramphenicol. At an optical density (600 nm) of 0.8–0.9 and prior to induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside and 20 mM L-arabinose, D-biotin and MnCl$_2$ were supplemented into the growth medium to 3 mg/liter and 0.1 mM, respectively. Cultures were induced for 16–20 h at 16 °C.

ΔBCΔBCCP RePC and all of its mutant forms were expressed in *E. coli* BL21Star(DE3) cells from a modified pET28a-His$_8$-TEV vector as described previously (18). Cells were cultured in M9 minimal medium containing 40 mg/liter kanamycin. When the culture reached an optical density (600 nm) of 0.8–1.0, MnCl$_2$ was supplemented in the growth medium to 0.1 mM, followed by induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 16–20 h at 16 °C.

All forms of RePC were purified using Ni$^{2+}$ affinity and anion exchange chromatography. Harvested cells were resuspended in Buffer
A, containing 20 mM Tris-HCl (pH 7.8), 5 mM imidazole, 200 mM NaCl, 0.5 mM EGTA, 6 mM 2-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 μM pepstatin A, and 5 μM epoxysuccinyl-L-leucylamido(4-guanido) butane. Cells were disrupted by sonication, and the cell lysate was cleared by centrifugation at 10 °C prior to loading onto a 10-ml Ni²⁺-nitrilotriacetic acid Profinity resin column (Bio-Rad). The enzyme was eluted from the column in Buffer A, with a gradient from 20 to 250 mM imidazole. Purified protein was pooled and dialyzed overnight against Buffer A at 4 °C. For all forms of ΔBCΔBCCP RePC, the Ni²⁺-nitrilotriacetic acid-purified preparation was incubated in Buffer A with His-tagged recombinant tobacco etch virus (rTEV) protease at 4 °C (40:1 molar ratio of ΔBCΔBCCP RePC to rTEV protease) to remove the N-terminal His₈ tag. After His₈ tag cleavage, the ΔBCΔBCCP RePC sample was reapplied to a Ni²⁺ affinity column to remove the cleaved His₈ tag and His-tagged rTEV protease. The His₈ tag cleavage and rTEV protease removal was >95% efficient as estimated by SDS-PAGE (data not shown). Cleaving the N-terminal His₈ tag of the full-length RePC with rTEV protease was unsuccessful, suggesting that the recognition sequence is inaccessible to the rTEV protease. Therefore, all full-length forms of RePC retained the recombinant N-terminal His₈ tag.

All purified protein samples were pooled and dialyzed in Buffer B, containing 20 mM triethanolamine (pH 8.0), 50 mM NaCl, 1 mM EGTA, and 2 mM DTT at 4 °C, in preparation for anion exchange chromatography. Protein was loaded on a 10-ml column volume of Q-Sepharose Fast Flow resin (GE Healthcare) and was sequentially eluted from the resin in Buffer B using a linear gradient from 50 mM to 1 mM NaCl. Fractions were pooled and dialyzed against a storage buffer consisting of 10 mM Tris-HCl (pH 7.8), 50 mM NaCl, 10 mM MgCl₂, 5% (v/v) glycerol, and 2 mM DTT. MgCl₂ was deliberately excluded from all buffers during purification of wild-type and mutant forms of ΔBCΔBCCP RePC. An Amicon stirred cell with a 30,000 molecular weight cut-off filter was used to concentrate protein preparations to a range of 3–12 mg/ml. Concentrated protein was flash frozen in liquid nitrogen prior to storage at −80 °C. Full-length RePC and ΔBCΔBCCP RePC enzyme concentrations were determined spectrophotometrically using the calculated molar extinction coefficient of 118,000 m⁻¹ cm⁻¹.
and 76,780 m$^{-1}$ cm$^{-1}$, respectively, at 280 nm (26). All full-length purified RePC proteins were >95% biotinylated, as revealed by an avidin-gel shift assay (data not shown) (20).

**Protein Crystallization**

Wild-type and the Y628A mutant form of ΔBCΔBCCP RePC were crystallized under similar conditions using the batch crystallization method under oil. Crystallization conditions for all x-ray crystal structures were nearly identical. For the ΔBCΔBCCP RePC structure co-crystallized with pyruvate, a protein solution consisting of 10 mg/ml ΔBCΔBCCP RePC and 30 mM pyruvate was mixed at a 1:1 ratio with a precipitant solution composed of 12–14% (w/v) PEG 8000, 100 mM BisTris (pH 6.0), 300–400 mM tetramethylammonium chloride, and 2% (v/v) glycerol. The Y628A mutant was co-crystallized with 50 mM pyruvate. A seed stock was created using the seed bead kit from Hampton Research (Aliso Viejo, CA). Briefly, a single apoprotein ΔBCΔBCCP RePC 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 (250 μm × 200 μm × 150 μm) formed within 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 10% (w/v) PEG 8000, 65 mM BisTris (pH 6.0), 200 mM tetramethylammonium chloride, 5% (v/v) glycerol, and 25 mM NaCl to a cryoprotectant solution consisting of 11.5% (w/v) PEG 8000, 90 mM BisTris (pH 6.0), 350 mM tetramethylammonium chloride, 20% (v/v) glycerol, and 25 mM NaCl and flash cooled in liquid nitrogen.

**Data Collection and Structure Determination**

X-ray diffraction data were collected at the Advanced Photon Source, beamline LS-CAT (Life Sciences Collaborative Access Team) 21-ID-F and 21-ID-G, on Rayonix MarMosaic 225 CCD and 300 CCD detectors, respectively. In all cases, the x-ray wavelength was tuned to 0.979 Å. Diffraction images were processed with the HKL2000 suite (27). In all cases, the diffraction data were anisotropic, with diffraction falling off in the b* direction relative to the a* and c* directions. This contributed
to the modest resolution limits and to the elevated Wilson $B$-value. Anisotropic data truncation using the UCLA MBI diffraction anisotropy server did not improve the electron density maps or the refinement statistics and was therefore not used for data processing. The structures were solved by molecular replacement using the CT + allosteric domains of the wild-type RePC enzyme (PDB code 2QF7) as the search model with the program Phaser (28). Following molecular replacement, translation/liberation/screw (TLS) refinements were performed using REFMAC (29–31). Initial $B$-factors were set at a constant value of 20.0 Å$^2$ prior to the start of refinement. Each monomer was treated as a rigid TLS group. TLS refinement improved the $2F_o - F_c$ maps and lowered the final $R$ and $R_{free}$ for each model by 1.5 and 3.0%, respectively, consistent with what is expected from using a single TLS group model (32). All four monomers in the asymmetric unit were restrained using non-crystallographic symmetry for all models during the entire refinement process. The models were extended by several rounds of manual model building with COOT (33) and successive refinements with REFMAC. Water molecules were added to the model in COOT with subsequent manual verification. In all cases, monomers A and B are packed more tightly in the asymmetric unit than monomers C and D. As a result, monomers A and B exhibit lower $B$-factors (Table 1) and are better defined by the electron density maps. All figures were generated from monomer A of the structure. Data collection and processing statistics are summarized in Table 1.
Pyruvate Carboxylation Assay

Pyruvate carboxylation activity was determined spectrophotometrically by measuring the conversion of oxaloacetate to malate using malate dehydrogenase, as described previously (22). Reaction assays were initiated by the addition of RePC (3–150 μg) in a 1-ml reaction volume at 25 °C. All enzyme substrates were maintained at saturation except for pyruvate, which was varied from 0 to 50 mm. Each reaction was performed in 0.1 M Tris-HCl (pH 7.8), 25 mM NaHCO₃, 2.5 mM MgATP, 7 mM MgCl₂, 0.25 mM acetyl-CoA, 0.24 mM NADH, and malate dehydrogenase (10 units).

Phosphorylation of MgADP Using Carbamoyl Phosphate

The phosphorylation of MgADP in the presence of carbamoyl phosphate was measured by following the production of ATP using a hexokinase/glucose-6-phosphate dehydrogenase coupled assay (20, 22, 34). Specific activities were determined at 25 °C in 3-ml reaction
mixtures containing 0.1 m Tris-HCl (pH 7.8), 7 mM MgCl₂, 3.5 mM MgADP, 0.25 mM acetyl-CoA, 20 mM carbamoyl phosphate, 0.4 mM glucose, 0.36 mM NADP, glucose-6-phosphate dehydrogenase (5 units), and hexokinase (2 units). The reaction was initiated by the addition of RePC (200 μg).

**Oxaloacetate Decarboxylation in the Presence and Absence of Oxamate**

The PC-catalyzed rate of oxaloacetate decarboxylation was determined by measuring the reduction of pyruvate to lactate using lactate dehydrogenase (20, 22, 35). Reactions were performed in 0.1 m Tris-HCl (pH 7.8), 0.25 mM acetyl-CoA, 0.24 mM NADH, and lactate dehydrogenase (10 units). For reactions catalyzed by ΔBCΔBCCP RePC, 1 mM oxaloacetate was added prior to initiation of the reaction with enzyme (400–900 μg). The half-life for the spontaneous decarboxylation of oxaloacetate is 2–6 h at room temperature (36). Therefore, oxaloacetate solutions were prepared fresh, kept on ice for the duration of the kinetic experiments, and added immediately prior to initiation of the reaction. The enzyme-catalyzed rate was obtained by subtracting the slow rate of non-enzymatic oxaloacetate decarboxylation from the rate measured in the presence of the enzyme. Biocytin or biotin was supplemented into these reactions at a final concentration of 5 mM. Reactions exhibited substrate inhibition in the presence of oxamate (0.5 mM); therefore, rates were determined at both 0.2 and 1 mM oxaloacetate. All reactions were conducted in a 1-ml reaction volume at 30 °C.

**Results and Discussion**

**Active Site Remodeling in the Carboxyltransferase Domain during Substrate Binding**

A small number of PC structures have been reported (15, 16, 18, 21), but direct comparisons between these structures have been complicated by differences in the source organism and in the set of bound ligands. To date, there are no directly comparable structures of
the CT domain of PC in the presence and absence of pyruvate. After screening several engineered constructs of RePC, it was determined that removing both the BC and BCCP domains (ΔBCΔBCCP RePC) rendered the enzyme more amenable to crystallization. The relative ease of crystallization afforded a unique opportunity to directly investigate the structural perturbations accompanying substrate binding in the CT domain of PC. Consequently, ΔBCΔBCCP RePC was crystallized with and without the bona fide substrate, pyruvate. The protein crystallized in the space group $P2_12_12_1$ under all co-crystallization conditions. The unit cell contains four monomers of ΔBCΔBCCP RePC, arranged as a dimer of dimers. In all structures, one homodimer is composed of chains A and C, whereas the second homodimer is composed of chains B and D. All four monomers of ΔBCΔBCCP RePC include a pyruvate molecule bound in the active site when co-crystallized with pyruvate. Representative electron density for chain A is presented in Fig. 1A.

**FIGURE 1.**
**Substrate binding leads to active site remodeling in the CT domain of RePC.** A, stereo view of representative electron density for the active site of ΔBCΔBCCP RePC co-crystallized with pyruvate. The $2F_0-F_c$ electron density map is contoured to 1.0 σ and is represented as a gray mesh. The $2F_0-F_c$ omit density map is contoured to 3.0 σ and is represented as a green mesh. The representative electron density is for monomer A. Electron density is not averaged. The modeled atoms correspond to the final submitted coordinates. B, stereo view of the active site from the CT domain upon pyruvate binding. The positioning of the orange loop is from the x-ray crystal structure devoid of pyruvate. Pyruvate is colored green. The multiple conformations for Arg$^{621}$ were modeled from RePC wild-type (PDB code 2QF7). C, structural alignment of the pyruvate-bound ΔBCΔBCCP RePC with the *H. sapiens* PC crystal structure (PDB code 3BG3; monomer B; r.m.s. deviation = 1.3 Å) reveals the expected positioning of biotin in the CT domain active site of RePC. In the closed conformation, Asp$^{590}$ and Tyr$^{628}$ form a surface to assist with biotin insertion into the active site.
As described previously, the CT domain architecture consists of a canonical $\alpha_8\beta_8$ TIM barrel fold with a large C-terminal funnel, composed of nine $\alpha$-helices, that leads into the active site at the mouth of the barrel. The active site is centered on a structurally conserved Lewis acid metal. Mutation of several conserved, metal-coordinating residues results in a loss of enzymatic activity in PC (37). PC enzymes from vertebrates predominantly bind Mn$^{2+}$, whereas yeast and bacteria prefer Zn$^{2+}$ (38). However, x-ray fluorescence analysis of the ΔBCΔBCCP RePC crystals indicates the presence of both Mn$^{2+}$ and Zn$^{2+}$ (data not shown), suggesting that RePC may be partially indiscriminate in its metal ion preference. Given that this may be an artifact of expression and purification from a heterologous host, further analyses of RePC purified from source are needed to unequivocally establish the physiological metal preference.
The structures in the presence and absence of pyruvate enable, for the first time, an analysis of conformational changes that accompany substrate binding in the CT domain of PC. The most pronounced difference between the structures determined in the presence and absence of pyruvate lies within a flexible loop (Arg⁶²¹–Asn⁶³⁰), which forms a portion of the CT domain active site. In the absence of substrate, the loop occupies an open conformation. The side chain of Tyr⁶²⁸ is oriented away from the metal center, and the guanidinium group of Arg⁶²¹ is disordered (Fig. 1B, orange). This disordered side chain is consistent with multiple conformations observed for Arg⁶²¹ in the higher resolution (2.0 Å) apo-structure of RePC (15). When pyruvate is bound, the active site loop adopts a closed conformation over the pyruvate binding site (Fig. 1B, cyan). The loop movement coincides with the formation of a salt bridge between the carboxyl moiety of pyruvate and the guanidinium group of Arg⁶²¹. Mutation of the equivalent residue to Arg⁶²¹ in Staphylococcus aureus PC (SaPC) resulted in a nearly complete loss of PC enzyme activity, indicating an important role for this residue in catalysis (21). The guanidinium group of Arg⁶²¹ is within hydrogen bonding distance to the backbone carbonyl oxygen of Val⁶²⁶ and Gly⁶²⁷ on the active site loop. These interactions are likely to stabilize the closed conformation of the loop. In the closed conformation, the tyrosyl moiety of Tyr⁶²⁸ rotates inward toward the active site, bringing it within hydrogen bonding distance of the carboxyl group from Asp⁵⁹⁰ that is located opposite the active site cleft. The positioning of Asp⁵⁹⁰ appears to be aided by the guanidinium side chain of Arg⁵⁹⁴, which is located within 2.5 Å of the carboxyl group of Asp⁵⁹⁰. Based on an overlay with the x-ray crystal structure of pyruvate-bound Homo sapiens PC, tethered biotin binds in the active site with its thiophene ring positioned directly above the aromatic ring of Tyr⁶²⁸ (Fig. 1C).

Taken together, these structures suggest that the CT domain active site receives carboxybiotin only after pyruvate is bound, when the active site loop has been remodeled into the closed conformation. These structural insights trace a direct connection between pyruvate binding and the subsequent formation of the biotin binding site in the CT domain and serve to significantly advance the description of how
PC and related biotin-dependent enzymes affect the control and coordination of biotin-dependent catalysis between remote active sites.

Substrate analogs that contain both the carboxyl and oxo groups of pyruvate, such as fluoropyruvate, hydroxypyruvate, oxamate, and glyoxylate, are inhibitors of the CT domain reaction of PC (reviewed in Ref. 39). Further, these compounds are sufficient to invoke the translocation and spontaneous decarboxylation of N-[14C]carboxybiotin in the CT domain of PC from sheep liver (24). However, propionate, lactate, and acetaldehyde, which lack either the carboxyl or oxo groups, do not invoke decarboxylation of N-[14C]carboxybiotin (24). The conformational remodeling observed in ΔBCΔBCCP RePC structures indicates that pyruvate binding leads directly to carboxybiotin insertion in the active site of the CT domain and explains why only close analogs of pyruvate are able to invoke carboxybiotin translocation and decarboxylation. X-ray crystal structures of ΔBCΔBCCP RePC co-crystallized with oxamate, oxalate, 3-bromopyruvate, or 3-hydroxypyruvate all occupy the binding site for pyruvate and promote the closed conformation of the CT domain.4

The conformational remodeling observed for the ΔBCΔBCCP RePC structures in the presence and absence of pyruvate is consistent with previously reported structures of PC. In the structure of SaPC co-crystallized with pyruvate (PDB code 3BG5), all four CT domain active sites of the tetramer have pyruvate bound and exhibit a closed conformation. Further, the BCCP domain is observed interacting with one of the four CT domains with biotin extending into the active site (16). In the only apparent contradiction of our hypothesis, the structure of T906A SaPC (SaPC Thr906 is equivalent to RePC Thr882; PDB code 3HBL) reveals biotin extending into the active site in the absence of pyruvate (21). Based on this observation, the authors suggest that pyruvate binding is not needed to facilitate BCCP-biotin docking in the active site. However, a lobe of well defined positive electron density occupies the binding site for pyruvate in the T906A SaPC structure and locks the guanidinium side chain of Arg644 (equivalent to RePC Arg621) into a fixed conformation, suggesting that T906A SaPC is not a true apo-structure. The unidentified molecule occupying this lobe of electron density promotes the closed
conformation, thereby accommodating BCCP-biotin insertion into the active site of the CT domain, consistent with our proposed model. Consequently, all reported structures of PC that reveal tethered biotin in the active site also have a ligand occupying the pyruvate binding site, consistent with our hypothesis.

Substrate-induced conformational remodeling also occurs in the α subunit of the oxaloacetate decarboxylase complex (ODC). Structural superposition of the Cα atoms of the CT-CT dimer from ΔBCΔBCCP RεPC with the CT-CT dimer of ODC from Vibrio cholerae (PDB code 2NX9) reveals a nearly identical active site architecture and dimer interface, with an r.m.s. deviation of 2.2 Å. The x-ray crystal structure of the CT domain from V. cholerae ODC was determined in the absence of substrate, and the active site displays an open conformation (40). Red edge excitation shift and FTIR data on V. cholerae ODC demonstrate that ligand binding restricts the tryptophan microenvironment and promotes structural changes in the CT domain (41). This suggests that structural rearrangements accompany substrate binding in the CT domain of homologous biotin-dependent enzymes.

**Steady State Kinetic Analysis of Asp$^{590}$ and Tyr$^{628}$ Mutations**

Structures of the RεPC CT domain suggest an important contribution from Asp$^{590}$ and Tyr$^{628}$ to catalysis. Both amino acid residues are conserved in all PC enzymes and in the CT domain of the homologous biotin-dependent enzymes, ODC and transcarboxylase. To determine the role of Asp$^{590}$ and Tyr$^{628}$ in the carboxylation of pyruvate, Y628F, Y628A, and D590A mutations were generated in RεPC by site-directed mutagenesis, and the modified enzymes were assessed for their catalytic activity. The $K_m$ for pyruvate, $k_{cat}$, and $k_{cat}/K_m$ were determined for the wild-type and mutant forms of RεPC. For the overall reaction, starting from HCO$_3^-$, MgATP, and pyruvate, all three mutations resulted in a pronounced effect on the $K_m$ for pyruvate, $k_{cat}$, and $k_{cat}/K_m$ for pyruvate carboxylation (Table 2). Removing the hydroxyl moiety from Tyr$^{628}$ (Y628F) resulted in a 7-fold reduction in $k_{cat}$, whereas the Y628A mutation resulted in a 780-fold
reduction in $k_{\text{cat}}$. The Tyr$^{628}$ mutations also resulted in a sizable increase in the $K_m$ for pyruvate, which contributed to the ∼80-fold and ∼20,000-fold decrease in catalytic efficiency for Y628F and Y628A, respectively. Mutating Asp$^{590}$ to Ala resulted in a 6-fold increase in $K_m$ for pyruvate and a 350-fold decrease in $k_{\text{cat}}/K_m$.

**TABLE 2**

Catalytic activities of full-length RePC mutants compared with wild type for the pyruvate carboxylation reaction. Reaction conditions were as follows: 100 mm Tris-HCl (pH 7.8), 25 °C, 25 mm NaHCO$_3^-$, 7 mm MgCl$_2$, 2.5 mm MgATP, 0.25 mm acetyl-CoA, pyruvate (0–50 mm). Errors reported are the S.E. values from the non-linear regression fit of the data to the Michaelis-Menten equation.

<table>
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<th>$k_{\text{cat}}$</th>
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<th>$K_m$ pyruvate</th>
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<tr>
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Although these mutations are located within the CT domain and disrupt the ability of the enzyme to carboxylate pyruvate, previous studies suggest that mutations in the CT domain can influence the rate of reactions in the neighboring BC domain (20). To evaluate the effects of these mutations on the BC domain activity, the specific activities for the phosphorylation of MgADP using carbamoyl phosphate as the phosphoryl donor were determined (**Table 3**). All mutations resulted in only a minor reduction in the ability of the BC domain to phosphorylate MgADP at saturating substrate concentrations.

**TABLE 3**

Catalytic activities of full-length RePC mutants compared with wild type for the ADP phosphorylation reaction using carbamoyl phosphate.

Reaction conditions were as follows: 100 mm Tris-HCl (pH 7.8), 25 °C, 7 mm MgCl$_2$, 3.5 mm MgADP, 0.25 mm acetyl-CoA, 20 mm carbamoyl phosphate.
The preceding results indicate that the catalytic role of both Asp\textsuperscript{590} and Tyr\textsuperscript{628} is exclusive to the CT domain reaction. To date, kinetic investigations of PC have demonstrated a significant effect of free biotin in the BC domain reaction (19, 23, 42). However, no such biotin dependence has been established in the CT domain reaction. To further investigate the role of Asp\textsuperscript{590} and Tyr\textsuperscript{628} in the CT domain reaction, the biotin-dependent and biotin-independent rates of oxaloacetate decarboxylation in the wild-type and mutant forms of \( \Delta \text{BC}\Delta \text{BCCP RePC} \) were measured. The biotin-dependent decarboxylation of oxaloacetate involves a carboxyl transfer from oxaloacetate to biotin, thus forming carboxybiotin (20, 25). However, PC also catalyzes the decarboxylation of oxaloacetate in the absence of biotin (22, 35). The truncation of the BC and BCCP domains from RePC generates a unique and simplified system to investigate the effect of free biotin on the rate of oxaloacetate decarboxylation.

At 1 mM oxaloacetate, each \( \Delta \text{BC}\Delta \text{BCCP RePC} \) mutation had a modest effect on the biotin-independent rate of oxaloacetate decarboxylation (Table 4). The Y628A \( \Delta \text{BC}\Delta \text{BCCP RePC} \) mutation represented the largest difference relative to wild type. The biotin-independent decarboxylation of oxaloacetate was measured by supplementing the reaction with biocytin and biotin. Biocytin, which includes a lysine side chain fused to the valerate side chain of biotin, is an ideal mimic of BCCP-biotin. The wild-type \( \Delta \text{BC}\Delta \text{BCCP RePC} \) rate of oxaloacetate decarboxylation increased by 5.6- and 1.9-fold in the presence of 5 mM biocytin and 5 mM biotin, respectively (Table 4). Notably, this biocytin-dependent rate enhancement is completely lost in the Tyr\textsuperscript{628} and Asp\textsuperscript{590} mutations of \( \Delta \text{BC}\Delta \text{BCCP RePC} \). Only the D590A \( \Delta \text{BC}\Delta \text{BCCP RePC} \) exhibited a rate enhancement (3.1-fold) in the presence of 5 mM biotin, probably because the mutation eliminated...
the charge repulsion between Asp<sup>590</sup> and the carboxylate moiety of free biotin. These results unequivocally demonstrate that Asp<sup>590</sup> and Tyr<sup>628</sup> are required to accommodate the binding of biotin in the CT domain active site.

**TABLE 4**

Biotin- and biocytin-dependent oxaloacetate decarboxylation activities for site-directed mutants of ΔBCΔBCCP RePC compared with wild-type ΔBCΔBCCP RePC. Reaction conditions were as follows: 100 mm Tris-HCl (pH 7.8), 30 °C, 1 mm oxaloacetate, 0.24 mm NADH.

<table>
<thead>
<tr>
<th></th>
<th>1 mm oxaloacetate, k&lt;sub&gt;cat&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; min&lt;sup&gt;−1&lt;/sup&gt;</th>
<th>+5 mm biotin</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;(+biotin)/k&lt;sub&gt;cat&lt;/sub&gt;(−biotin)</th>
<th>+5 mm biocytin</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;(+biocytin)/k&lt;sub&gt;cat&lt;/sub&gt;(−biocytin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type CT</td>
<td>0.46 ± 0.007</td>
<td>0.48 ± 0.01</td>
<td>1.0</td>
<td>2.56 ± 0.006</td>
<td>5.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y628A</td>
<td>0.38 ± 0.002</td>
<td>0.41 ± 0.01</td>
<td>1.1</td>
<td>0.41 ± 0.007</td>
<td>1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T628A</td>
<td>0.33 ± 0.01</td>
<td>0.37 ± 0.006</td>
<td>1.1</td>
<td>0.36 ± 0.006</td>
<td>1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T628A + D590A</td>
<td>0.38 ± 0.01</td>
<td>1.16 ± 0.03</td>
<td>2.2</td>
<td>0.40 ± 0.006</td>
<td>1.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Specific activities were determined in triplicate, and the error reported is the S.D. value of the three determinations.

Oxaloacetate decarboxylation was also assayed for the wild-type and mutant forms of full-length RePC in the presence and absence of oxamate (Table 5). Oxamate, a structural analog of pyruvate, serves as a carboxyl acceptor from carboxybiontin in the CT domain; in the presence of oxamate, the decarboxylation of oxaloacetate proceeds through a double-displacement reaction mechanism with biotin acting to shuttle CO₂ from oxaloacetate to oxamate (43). Wild-type RePC exhibits a k<sub>cat</sub> of 1.23 min<sup>−1</sup> and 1.29 min<sup>−1</sup> for the decarboxylation of oxaloacetate when assayed at 1 and 0.2 mM oxaloacetate, respectively. At higher concentrations of oxaloacetate, the reaction is subject to substrate inhibition in the presence of oxamate. Therefore, the oxamate-induced decarboxylation of oxaloacetate was measured at 0.2 mM oxaloacetate. In the presence of 0.5 mM oxamate, the rate of oxaloacetate decarboxylation is enhanced 3-fold for wild-type RePC. However, this rate enhancement is lost in the Tyr<sup>628</sup> and Asp<sup>590</sup> mutants. These results provide additional evidence that Tyr<sup>628</sup> and Asp<sup>590</sup> are critical for the biotin dependence of RePC. The combined kinetic data are fully consistent with our model; substrate binding in the CT domain initiates the closure of the active site loop (Arg<sup>621</sup>–Asn<sup>630</sup>), which is necessary for the formation of the biotin binding pocket through the conserved interaction between Tyr<sup>628</sup> and Asp<sup>590</sup>. 
TABLE 5
Catalytic activities of site-directed mutants of full-length RePC compared to wild-type for the oxamate-induced decarboxylation of oxaloacetate. Reaction conditions were as follows: 100 mm Tris-HCl (pH 7.8), 30 °C, 0.2 and 1 mm oxaloacetate, 0.5 mm oxamate, 0.25 mm acetyl-CoA.

<table>
<thead>
<tr>
<th></th>
<th>1 mm oxaloacetate, $k_{\text{cat}}$ (min$^{-1}$)</th>
<th>0.2 mm oxaloacetate, $k_{\text{cat}}$ (min$^{-1}$)</th>
<th>0.2 mm oxaloacetate + 0.5 mm oxamate $k_{\text{cat}}$ (min$^{-1}$)</th>
<th>$k_{\text{cat}}$ (oxamate)/$k_{\text{cat}}$ (−oxamate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1.23 ± 0.06</td>
<td>1.78 ± 0.02</td>
<td>3.52 ± 0.2</td>
<td>3.0</td>
</tr>
<tr>
<td>Y628F</td>
<td>0.97 ± 0.05</td>
<td>0.87 ± 0.06</td>
<td>0.99 ± 0.03</td>
<td>1.1</td>
</tr>
<tr>
<td>Y628A</td>
<td>0.79 ± 0.04</td>
<td>0.56 ± 0.01</td>
<td>0.89 ± 0.01</td>
<td>1.9</td>
</tr>
<tr>
<td>D590A</td>
<td>0.62 ± 0.02</td>
<td>0.45 ± 0.01</td>
<td>0.81 ± 0.02</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* Specific activities were determined in triplicate, and the error reported is the S.D. value of the three determinations.

The X-ray Crystal Structure of Y628A ΔBCΔBCCP RePC

In order to further evaluate the role of Tyr$^{628}$ in catalysis, we crystallized the Y628A ΔBCΔBCCP RePC mutant in the presence of pyruvate. The Y628A mutant has exactly the same overall conformation as the wild-type ΔBCΔBCCP RePC. Structural superposition of the Cα atoms from the Y628A ΔBCΔBCCP RePC dimer with the dimers of ΔBCΔBCCP RePC co-crystallized in the presence and absence of pyruvate yields an r.m.s. deviation of 0.2 and 0.3 Å, respectively.

Of the four monomers in the asymmetric unit, only one member of each homodimer pair (monomer A of CT dimer 1 and monomer B of CT dimer 2) had pyruvate bound in the active site. Neither pyruvate nor the guanidinium portion of Arg$^{621}$ could be resolved in the active site of monomers C and D. The active sites from monomers A and B are equivalent to the active site for ΔBCΔBCCP RePC co-crystallized with pyruvate, and the binding orientation of pyruvate in the active sites from monomers A and B is unperturbed relative to the wild-type enzyme (Fig. 2). The salt bridge interaction between the carboxyl moiety of pyruvate and the guanidinium side chain of Arg$^{621}$ is retained, despite the loop being 2 Å further displaced from the metal center. However, the positioning of the loop resembles the open conformation observed for ΔBCΔBCCP RePC in the absence of pyruvate.
FIGURE 2
The interaction between Arg$^{621}$ and pyruvate is unperturbed in the Y628A ΔBCΔBCCP RePC crystal structure. Shown is a stereo view of the simulated annealing omit map contoured at 3.0 σ for Arg$^{621}$ and pyruvate (rose). The positioning of pyruvate and the loop when the active site is in the closed conformation is represented in cyan.

The structure of Y628A ΔBCΔBCCP RePC further supports a role for Tyr$^{628}$ in stabilizing the closed conformation of the active site. Upon substrate binding, Tyr$^{628}$ is repositioned such that the hydroxyl moiety is within hydrogen bonding distance of Asp$^{590}$ (see above). In the Y628A mutation, the interaction with Asp$^{590}$ is destroyed, and the active site binds pyruvate but remains in the open conformation. The biotin binding pocket cannot form in the open conformation, and carboxybiotin insertion is prevented. The Y628A structure, therefore, is fully consistent with the kinetic data showing that this mutant is not activated by biocytin, biotin, or BCCP-biotin (Tables 4 and 5).

Our kinetic analyses reveal a clear discrepancy between the Y628F and Y628A mutants of RePC. The Y628F RePC mutant is capable of carboxylating pyruvate 250-fold more efficiently than the Y628A RePC mutant, suggesting that the aromatic portion of Tyr$^{628}$ plays a specific role in catalysis. When biotin is bound in the active site of PC, the sulfur atom of the thiophene ring is positioned over the aromatic π-electron cloud of Tyr$^{628}$ (16). There exists clear structural evidence for the preferential localization of divalent sulfur (−CH$_2$−S−CH$_3$) at the edge and slightly above the plane of aromatic rings, both in protein stabilization and protein-ligand complexes (44, 45). A single sulfur-
aromatic interaction is estimated to contribute 1–2 kcal/mol in stabilization energy \((46)\). Given that there are few specific interactions serving to facilitate biotin binding in the CT domain active site \((16)\), the sulfur/π interaction between biotin and Tyr\(^{628}\) is expected to contribute to carboxybiotin binding in the active site.

**A Surprising Symmetrical Tetramer from R. etli PC**

Complete structures of PC have been determined for the enzyme cloned from *R. etli* and *S. aureus* \((15, 16, 18, 21)\). In all cases, the quaternary structure reveals a tetrameric rhombohedron that is stabilized at the corners through homodimer interactions between the BC and CT domains. However, despite \(\sim50\%\) sequence identity between *SaPC* and *RePC*, there have been striking differences in the overall arrangement of the tetramers; the opposing faces of the enzyme are highly asymmetrical in *RePC*, whereas they are nearly symmetrical in *SaPC* \((15, 16, 18, 21)\). The basis for this striking deviation in quaternary structure remains unclear.

Truncation of the BC and BCCP domains in *RePC* offered an opportunity to investigate the contribution of the CT domain to the quaternary arrangement of the enzyme. To our surprise, two ΔBCΔBCCP *RePC* dimers interact via their allosteric domains to form a symmetrical tetramer \((Fig. 3)\). The contacts between these opposing allosteric domains are minimal, contributing only \(\sim320\ \AA^2\) of buried surface area compared with \(\sim1400\ \AA^2\) of buried surface area for the CT-CT homodimer interface. The most prominent contact between allosteric domains is a parallel π-stacking interaction between Phe\(^{1051}\) residues on opposing chains. This same interaction has been shown to be essential for tetramerization in both *H. sapiens* PC and *SaPC* \((16)\). The symmetrical arrangement of the ΔBCΔBCCP *RePC* tetramer is in good agreement with what has been observed both in *SaPC* and in a BC domain truncation of *H. sapiens* PC \((Fig. 3, A and B)\) but is in sharp contrast with the asymmetrical tetramer of full-length *RePC* \((Fig. 3C)\). The CT-CT dimer structure is identical between the truncated and full-length *RePC*; structural superposition of the Cα atoms of the CT-CT dimer from ΔBCΔBCCP *RePC* with wild-type, full-length *RePC* reveals an r.m.s. deviation of 0.3 Å. Therefore, the asymmetry observed in
full-length RePC is likely to originate in the BC domain, which occupies a much greater range of conformations than the CT domain in all x-ray crystal structures of PC (15, 16, 18, 21).

FIGURE 3
The structure of the BC and BCCP domain truncation of RePC reveals a symmetrical tetramer. The green and cyan domains represent the CT domain, whereas the blue and magenta domains represent the allosteric/pyruvate carboxylase tetramerization (PT) domains. The green and blue domains are located on the top face of the tetramer, whereas the pink and cyan domains are on the bottom face. A, structural alignment between the chain A and C homodimer of ΔBCΔBCCP RePC and the chain A and D homodimer of ΔBC human pyruvate carboxylase (brown-colored, semitransparent ribbons; PDB code 3BG3). B, structural alignment between the chain A and C homodimer of ΔBCΔBCCP RePC and the chain A and D homodimer of the allosteric + CT domains of the full-length S. aureus PC enzyme tetramer (yellow-colored, semitransparent ribbons; PDB code 3BG5). C, structural alignment between the chain A and C homodimer of the ΔBCΔBCCP RePC and the chain A and B homodimer of the allosteric + CT domains of the full-length RePC enzyme tetramer (rose-colored, semitransparent ribbons; PDB code 2QF7).

Because the only data on the quaternary arrangement of PC come from x-ray or cryo-EM data, it is not yet clear whether RePC and SaPC are locked in these contrasting conformations or whether these structures are reflective of a range of conformations in solution. The surprising finding that RePC crystallizes as a symmetrical tetramer in the absence of the BC and BCCP domains demonstrates that RePC can access a wider range of conformations than previously recognized.
In summary, we report structural and kinetic evidence for a substrate-induced biotin binding pocket in the CT domain of PC. All biotin-dependent enzymes must coordinate two independent reactions in physically separate active sites. The translocation of tethered biotin links these activities. Evidence is emerging that the individual active sites are structured to control biotin access, thereby avoiding abortive decarboxylation and ensuring efficient coupling between the BC and CT domain reactions. According to our model, biotin is unable to bind in the CT domain in the absence of a carboxyl group acceptor. In this way, PC has evolved a mechanism to avoid the energetically wasteful decoupling of ATP hydrolysis in the BC domain from pyruvate carboxylation in the CT domain at physiological substrate concentrations. Based on sequence conservation and similar structural and biophysical evidence in ODC and transcarboxylase, we expect that this general mechanism is applicable to the wider family of biotin-dependent enzymes.

Acknowledgments

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The atomic coordinates and structure factors (codes 4JX4, 4JX5, and 4JX6) have been deposited in the Protein Data Bank (http://wwpdb.org/).

*4A. D. Lietzan and M. St. Maurice, unpublished data.
The abbreviations used are:

PC pyruvate carboxylase  
BC biotin carboxylase  
BCCP biotin carboxyl carrier protein  
BisTris 2-[(bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol  
CT carboxyltransferase  
ODC oxaloacetate decarboxylase complex  
RePC and SaPC R. etli and S. aureus pyruvate carboxylase, respectively  
rTEV protease recombinant tobacco etch virus protease  
TLS translation/libration/screw  
r.m.s. root mean square  
PDB Protein Data Bank.

References