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Functionally Diverse Biotin-dependent Enzymes with Oxaloacetate Decarboxylase Activity

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Abstract

Biotin-dependent enzymes catalyze carboxylation, decarboxylation and transcarboxylation reactions that participate in the primary metabolism of a wide range of organisms. In all cases, the overall reaction proceeds via two half reactions that take place in physically distinct active sites. In the first half-reaction, a carboxyl group is transferred to the 1-N' of a covalently tethered biotin cofactor. The tethered carboxybiotin intermediate subsequently translocates to a second active site where the carboxyl group is either transferred to an acceptor substrate or, in some bacteria and archaea, is decarboxylated to biotin and CO_2 in order to power the export of sodium ions from the cytoplasm. A homologous carboxyltransferase domain is found in three enzymes that

catalyze diverse overall reactions: carbon fixation by pyruvate carboxylase, decarboxylation and sodium transport by the biotin-dependent oxaloacetate decarboxylase complex, and transcarboxylation by transcarboxylase from *Propionibacterium shermanii*. Over the past several years, structural data have emerged which have greatly advanced the mechanistic description of these enzymes. This review assembles a uniform description of the carboxyltransferase domain structure and catalytic mechanism from recent studies of pyruvate carboxylase, oxaloacetate decarboxylase and transcarboxylase, three enzymes that utilize an analogous carboxyltransferase domain to catalyze the biotin-dependent decarboxylation of oxaloacetate.

Keywords

Enzyme, Biotin, Decarboxylation, Pyruvate carboxylase, Transcarboxylase, Oxaloacetate decarboxylase

Introduction

In 1933, Burk and colleagues described an accessory factor isolated from egg yolk that was essential for the growth of the nitrogen-fixing bacterium, *Rhizobium* [1]. Three years later, Kögl and Tonnis isolated a crystalline 'Bios' factor from duck egg yolks that they called biotin [2]. Shortly thereafter, biotin was recognized as an essential growth factor common to bacteria, yeast and mammals (reviewed in [3]). The structure of biotin was soon chemically deduced (Fig. 1; [4]) and it was shown to be involved in carboxylation reactions, providing a clue into its role in cellular physiology [5]. Eventually, biotin was found to be covalently tethered to and necessary for catalysis in acetyl coenzyme A (CoA)[1] carboxylase [6]. This critical observation established a biological role for biotin in CO_2 fixation and resulted in the discovery of a family of enzymes known today as the biotin-dependent enzymes.

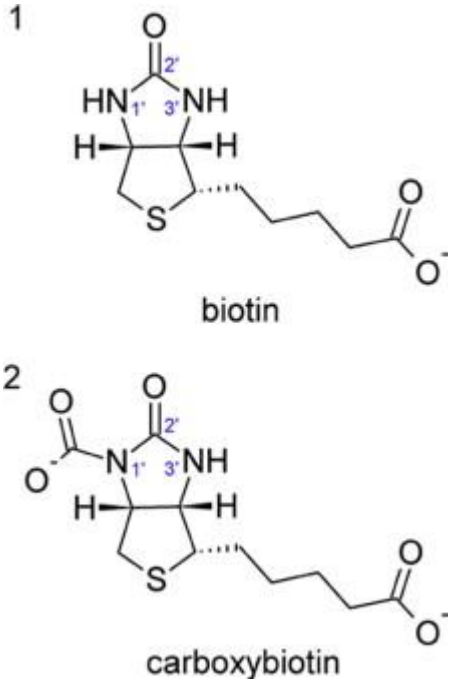

Fig. 1. Chemical structure of biotin and *N*-carboxybiotin. (1) Biotin, also known as vitamin H, vitamin B_7 or coenzyme R, is composed of a valerate side chain tethered to a bicyclic ring. The bicyclic ring consists of a ureido ring fused to a tetrahydrothiophene ring. (2) *N*-carboxybiotin is the reaction intermediate for all biotin-dependent enzymes. Biotin is carboxylated at the N-1 position.

In recent years, the description of structure and function in the biotin-dependent enzymes has advanced considerably, primarily as a result of an influx of new X-ray crystal structures. Two recent reviews [7], [8] have

summarized these structural advances, particularly in the Class I biotin-dependent enzymes, and the reader is referred to these sources for a complete and recent description of the carboxylase class of biotin-dependent enzymes. This review focuses, instead, on the structure and function of the carboxyltransferase domain from three enzymes that encompass the three classes of the biotin-dependent family: pyruvate carboxylase, oxaloacetate decarboxylase and transcarboxylase. These three enzymes catalyze very different overall reactions, but structural and kinetic data compiled over the past 10 years have provided significant insights into common active site features, shared aspects of chemical mechanism and similarities in the interactions between the carboxyltransferase domain, the biotin cofactor and associating functional domains.

Biotin-dependent enzymes

The biotin-dependent enzymes are present across all three domains of life, and phylogenetic analyses of the individual domains suggest an ancient evolutionary origin [9]. These enzymes are composed of two distinct active sites, with a covalently tethered biotin cofactor traveling between active sites via a so-called swinging-arm mechanism [10]. All enzymes in this family must coordinate the biotin-dependent transfer of a carboxyl moiety between remote active sites by way of two independent half-reactions. As such, they require multiple catalytic domains to accomplish catalysis.

Biotin serves as the conduit for carboxyl transfer and is carboxylated at the 1'-*N* position in the first active site (Fig. 1; [11], [12], [13]). The resulting carboxybiotin product is relatively stable, with a half-life exceeding 100 min at pH 8 [14]. The stability of 1'-*N*-carboxybiotin is critical, since energy is expended in carboxylating biotin and because carboxybiotin must physically translocate to a second active site prior to transferring the carboxyl moiety to an acceptor substrate.

The biotin cofactor is covalently attached, via an amide linkage, to the ε-amino group of a specific, conserved lysine in the biotin carboxyl carrier protein (BCCP) domain by the enzyme biotin protein ligase [15]. Several NMR and X-ray crystal structures of the BCCP domain reveal two sets of four antiparallel β-strands arranged around an approximate twofold axis [16], [17], [18], [19]. The site of biotinylation is located on a hairpin turn that connects two β-strands near the C-terminal end of the domain. In most cases, the modified lysine residue is bracketed within a conserved sequence consisting of Ala–Met–Lys–Met. In pyruvate carboxylase and transcarboxylase, this identity extends to Ala–Met–Lys–Met–Glu–Thr. The BCCP domain physically translocates between catalytic domains and assists in inserting biotin into the individual active sites. The primary sequence and tertiary structure for the C-terminal ∼80 amino acids of the BCCP domain is conserved for all enzymes within the family, suggesting that they are descended from a common ancestor [20]. In *Escherichia coli* acetyl-CoA carboxylase, however, the BCCP subunit includes an additional N-terminal domain of ∼80 amino acids which may serve to impede the catalytic half-reactions prior to assembly of the complete enzyme complex [21], [22].

The biotin-dependent enzymes are divided into three classes, depending on the overall reaction that they catalyze [23]. These are briefly described below.

Class I – carboxylases

Class I enzymes are unique within the biotin-dependent family in catalyzing carbon fixation. On the basis of chemical reactivity, enzymes involved in carbon fixation would be predicted to favor nucleophilic attack on the electrophilic carbon of carbon dioxide. However, at physiological pH and temperature, the concentration of dissolved carbon dioxide is twenty times less than bicarbonate ion (10 μM vs. 200 μM), making dissolved carbon dioxide availability low by comparison [24]. While there are enzymes that utilize dissolved carbon dioxide as a substrate (ribulose-1,5-bisphosphate carboxylase oxygenase, or RuBisCO, is a well-known example), these

enzymes often suffer from low catalytic activity. Biotin-dependent carboxylases have evolved to effectively utilize the more abundant bicarbonate ion for carbon fixation [25], [26].

The biotin-dependent carboxylases include pyruvate carboxylase (EC 6.4.1.1), acetyl CoA carboxylase (EC 6.4.1.2), propionyl CoA carboxylase (EC 6.4.1.3), 3-methylcrotonyl CoA carboxylase (EC 6.4.1.4), geranyl CoA carboxylase (EC 6.4.1.5), and urea carboxylase (EC 6.3.4.6) (Fig. 2). All enzymes within this class use a common mechanism to carboxylate biotin in the structurally conserved biotin carboxylase (BC) domain. A detailed description of the structure and mechanism of the BC domain of the Class I biotin-dependent enzymes is provided in a recent review [7]. In the presence of two metal ions, typically Mg^{2+}, ATP is cleaved to activate bicarbonate for the subsequent carboxylation of biotin (Fig. 3). The carboxyl moiety is then transferred from carboxybiotin to an acceptor substrate in a completely separate carboxyltransferase domain active site. Many of these Class I enzymes use a common mechanism and a conserved carboxyltransferase domain to act on a variety of thioester substrates [7] but a subset within this class, namely pyruvate carboxylase and urea carboxylase, do not act on thioester substrates and their carboxyltransferase domains differ substantially in structure and mechanism from each other and from the other enzymes in this class.

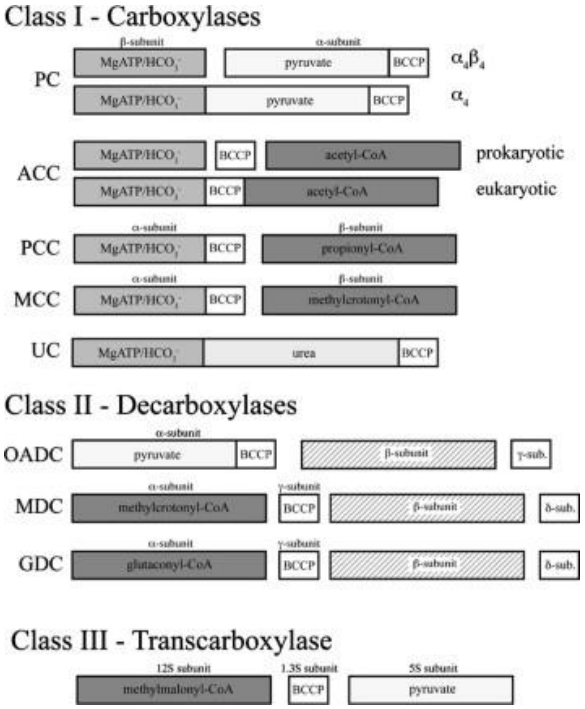

Fig. 2. Schematic diagram of the biotin-dependent enzyme family showing the arrangement of catalytic and biotin carrier domains. The biotin-dependent enzyme family is subdivided into three classes. The *Class I carboxylases* include pyruvate carboxylase (PC), acetyl-CoA carboxylase (ACC), propionyl-CoA carboxylase (PCC), methylcrotonyl-CoA carboxylase (MCC), urea carboxylase (UC) and geranyl-CoA carboxylase (not shown). Within this class, both PC and ACC are further subdivided into distinct structural arrangements. As described in Section 2.1, the majority of PC enzymes are of the α_4 form, with all domains arranged on a single polypeptide chain, while a few “subunit type” PC enzymes are composed of separate α- and β-subunits. The ACC enzymes of prokaryotic origin are composed of individual subunits while eukaryotic ACC is a multifunctional enzyme, with all domains originating from a single polypeptide chain. The *Class II decarboxylases* include oxaloacetate decarboxylase (OADC), methylcrotonyl-CoA decarboxylase (MDC), glutaconyl-CoA decarboxylase (GDC) and malonate decarboxylase (not shown). The *Class III transcarboxylase* consists of a single enzyme, transcarboxylase from *P. shermanii*, described in the section titled "Transcarboxylase". The biotin carboxyl carrier protein domain is abbreviated as BCCP. The domains are colored to highlight the homology among the individual domains.

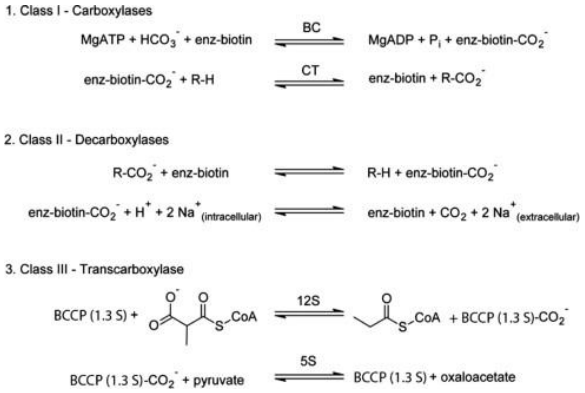

Fig. 3. Reaction schemes for the various classes of enzymes within the biotin-dependent enzyme family. The general reaction scheme for each biotin-dependent enzyme class is represented. The classes are as follows: (1) Class I – carboxylases; (2) Class II – decarboxylases; (3) Class III – transcarboxylase.

Class II – decarboxylases

Class II enzymes facilitate sodium transport from the cytoplasm to the periplasm in some archaea and anaerobic bacteria (Fig. 3). The enzymes included in this class are oxaloacetate decarboxylase (ODC; EC 4.1.1.3), methylmalonyl CoA decarboxylase (EC 4.1.1.41) and glutaconyl CoA decarboxylase (EC 4.1.1.70) (Fig. 2). A biotin-dependent malonate decarboxylase (EC 4.1.1.89) has also been described that proceeds through the formation of a malonyl-thioester with a specific acyl carrier protein (ACP) [27], [28]. Decarboxylating the β-keto acid of oxaloacetate or the thioester of methylmalonyl CoA, glutaconyl CoA or malonyl-S-ACP affords the necessary free energy to pump sodium ions across the lipid bilayer [28], [29], [30], [31], [32]. The resulting sodium gradient drives the synthesis of ATP, solute transport and motility in these organisms [33]. This class of biotin-dependent enzymes does not transfer a carboxyl moiety to an acceptor substrate. Instead, carboxybiotin transits to the membrane-bound β-subunit where it is decarboxylated to biotin and CO_2 in a reaction that consumes a periplasmic proton and is coupled to Na^+ translocation from the cytoplasm to the periplasm.

Class III – transcarboxylase

The only enzyme currently described in Class III is transcarboxylase from *Propionibacterium shermanii* (EC 2.1.3.1; Fig. 2). Transcarboxylase transfers a carboxyl moiety from methylmalonyl CoA to pyruvate, thus resulting in the formation of propionyl CoA and oxaloacetate [34]. Transcarboxylase is unique among the biotin-dependent enzymes in that it catalyzes carboxyl transfer between two organic molecules. The enzyme utilizes two separate carboxyltransferase domains to accomplish this transfer (Fig. 3).

Biotin-dependent enzymes with oxaloacetate decarboxylation activity

Biotin-dependent enzymes catalyze carboxylation, decarboxylation and transcarboxylation reactions. Despite this diversity of reactions, homologous carboxyltransferase domains appear in enzyme members from all three classes. Phylogenetic analysis suggests that the carboxyltransferase domain of pyruvate carboxylase originated in an ancestral enzyme that predates the last universal common ancestor and that this domain was subsequently usurped for other activities, such as in the oxaloacetate decarboxylase complex of anaerobic bacteria [9]. Before describing the detailed structure and mechanism of this carboxyltransferase domain, a brief description of pyruvate carboxylase (PC), oxaloacetate decarboxylase (OADC) and transcarboxylase (TC) will serve to introduce the general properties and the metabolic context of three biotin-dependent enzymes that catalyze distinct overall reactions linked through a common biotin-dependent carboxyltransferase mechanism.

Pyruvate carboxylase

Woronick and Johnson first identified an ATP-dependent CO_2-fixing system in cell-free extracts from *Aspergillus niger* [35]. This novel reaction was postulated to result in the production of oxaloacetate from pyruvate, ATP and CO_2. These initial observations swiftly lead to the discovery of an enzyme in avian and bovine liver tissue which catalyzes the production of oxaloacetate in an ATP-, CO_2-, pyruvate-, Mg^{2+}-, and biotin-dependent manner (Fig. 31; [36]). This novel enzyme was designated pyruvate carboxylase (PC). To date, PC enzymes have been identified across a wide range of organisms including bacteria, fungi, monocot/dicot plants, invertebrates, and vertebrates [37], [38]. The structure and mechanism of PC has been the subject of many detailed and comprehensive reviews [39], [40], [41], [42] and only a cursory introduction to the metabolic context and the overall structure is provided here.

PC plays an important role in supplying oxaloacetate to the tricarboxylic acid (TCA) cycle in order to replenish intermediates lost to competing biosynthetic pathways. Thus, PC activity directly influences the availability of metabolites for fatty acid and amino acid biosynthesis. Human congenital deficiencies in PC may result in severe lactic acidemia, improper psychomotor development, mental retardation and, sometimes, death within a few weeks (reviewed in [43]). These severe phenotypes can, in part, be attributed to reduced γ-aminobutyric acid and glutamate production, essential neurotransmitters needed for neuronal development [44]. Additionally, several other disease states, including type-2 diabetes and tumor proliferation, are subject to aberrant PC activities and/or protein levels [45], [46], [47].

The native, active form of PC is a tetramer. However, two distinct variants of the tetramer exist, depending on the source organism. For most PC enzymes, the functional domains are arranged on a single ∼1200 amino acid polypeptide chain with a molecular weight of ∼120–130 kDa. Four identical polypeptide chains associate together to form a homotetramer of ∼500 kDa that constitutes the α_4 form (Fig. 2). The less common form is the $\alpha_4\beta_4$ form (or "subunit type"), which is observed primarily in archaea [48], [49] along with some bacterial species such as *Pseudomonas* citronellolis [50], [51] and *Aquifex aeolicus* [52]. This tetrameric form consists of two distinct polypeptide chains: a ∼55 kDa polypeptide (β) chain which has MgATP-dependent biotin carboxylation activity and a ∼70 kDa polypeptide (α) chain which includes an N-terminal carboxyltransferase domain and a C-terminal BCCP domain (Fig. 2). Four α and four β polypeptide chains associate together to form an active heterooctamer. To date, structural studies of the $\alpha_4\beta_4$ PC enzymes have been limited. However, the quaternary configuration of the $\alpha_4\beta_4$ PC enzyme from *P. citronellolis* is proposed to be similar to that of α_4 type PC enzymes [50].

The first X-ray crystal structure of a complete biotin-dependent enzyme was reported for the α_4-type PC from *Rhizobium etli* [53]. The *R. etli* PC structure was closely followed by the report of the holoenzyme structure of PC from *Staphylococcus aureus* [54]. Together, these X-ray analyses unveiled the quaternary arrangement of PC and revealed the relative disposition of the individual catalytic domains. The monomers of the α_4 PC tetramer are arranged on two distinct layers, running perpendicularly between the two layers and oriented antiparallel to each other on opposite sides of the tetramer (Fig. 4). Minimal direct contacts are observed between monomers located on the same layer. The tetramer is held together at the corners by BC–BC and CT–CT dimerization interfaces. From the arrangement of the catalytic domains and analysis of mixed hybrid tetramers, it was determined that BCCP-biotin physically moves by ∼50–70 Å from the BC domain on one polypeptide chain to the carboxyltransferase domain on a neighboring polypeptide chain (Fig. 4). This initial model for catalysis was confirmed by additional structures that captured the BCCP domain physically interacting with the carboxyltransferase domain of a neighboring polypeptide chain [54], [55], [56]. These data served to explain the long-standing observation that PC does not exhibit pyruvate carboxylation activity in its dimeric or monomeric states [57], [58], [59].

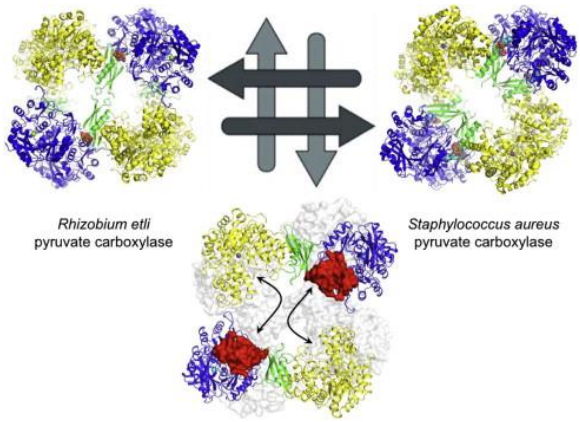

Fig. 4. Quaternary structure and intermolecular reaction mechanism of pyruvate carboxylase. The PC tetramer is arranged in two distinct layers, with the monomers running antiparallel on each layer and perpendicularly between the layers. Both *R. etli* (pdb i.d. 2QF7) and *S. aureus* (pdb i.d. 3BG5) primarily associate at the dimer interface between the biotin carboxylase (blue) and carboxyltransferase (yellow) domains. *S. aureus* PC maintains contacts between the allosteric (green) domains from opposite layers of the tetramer. This interaction is not observed in *Re*PC. For clarity and to enable comparison, the BCCP domain is omitted from the top illustrations of the *R. etli* and *S. aureus* PC tetramers. The bottom tetramer illustrates the translocation of the BCCP domain (red) between the biotin carboxylase and carboxyltransferase domain active sites on opposing monomers of the *R. etli* PC enzyme (based on pdb i.d. 2QF7). The distance between active sites of opposing monomers on the same face is ~60 Å. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In the overall reaction catalyzed by PC, the carboxyltransferase domain catalyzes the second half-reaction. After the tethered biotin cofactor is carboxylated in the BC domain with the concomitant cleavage of ATP, the BCCP domain translocates to the carboxyltransferase domain, where the carboxyl group is transferred from carboxybiotin to pyruvate, forming oxaloacetate. The reaction equilibrium in PC strongly favors this forward reaction. However, the carboxyltransferase domain half-reaction is routinely assayed by following the decarboxylation of oxaloacetate, and the overall reaction can also be run in the reverse direction, starting with oxaloacetate, ADP and P_i [60], [61], [62], [63]. Thus, while the carboxyltransferase domain of PC normally catalyzes the carboxylation of pyruvate, it may also be considered a biotin-dependent oxaloacetate decarboxylase.

Oxaloacetate decarboxylase complex

In a series of papers published in the early 1980s, Peter Dimroth demonstrated that the membrane-bound oxaloacetate decarboxylase complex (OADC) of *Klebsiella aerogenes* catalyzes the biotin-dependent decarboxylation of oxaloacetate, while also serving as a primary Na^+ pump [64], [65], [66]. OADC plays an essential role in the citrate or tartrate fermentation pathways of certain archaea and bacteria, contributing to the generation of an electrochemical gradient of Na^+ ions along with one mol of ATP per mol of citrate/tartrate. The resulting Na^+ gradient is used to power the import of nutrients and the synthesis of ATP. The structure and mechanism of OADC was last reviewed by Buckel [29] and by Dimroth et al. [31], both in 2001. Since then, only a handful of publications have focused on OADC, but several of these served to significantly advance the description of the structure and mechanism of this enzyme. Most notably, Studer et al. reported the X-ray crystal structure of the carboxyltransferase domain of OADC in 2007 [67], while, more recently, Balsera, Buey and Li used homology modeling and SAXS data to propose an updated model for the OADC quaternary structure [68].

OADC is typically composed of three subunits: α, β and γ Fig. 2 and Fig. 3. The ~65 kDa hydrophilic α-subunit consists of an N-terminal carboxyltransferase domain connected to a C-terminal BCCP domain. The α-subunit is a peripheral membrane protein on the cytosolic side of the membrane, where it associates with β- and γ-subunits that are embedded in the membrane. While the exact multimer size may vary depending on the source organism, the OADC complex from *Vibrio cholerae* is a tetramer [69] and the *α*:*β*:*γ* stoichiometry has long been assumed to be 1:1:1 based on the original analysis performed on the enzyme from *K. aerogenes* [70]. However, according to the X-ray crystal structure and solution studies, in the absence of β- or α-subunits, the γ-subunit forms a homodimer through a dimerization interface in the carboxyltransferase domain [67], [68] while it forms a larger multimeric complex in the presence of the γ-subunit, with a predicted *α*:*β*:*γ* stoichiometry of 4:2:2 [68]. The ~45 kDa β-subunit is an integral membrane protein with nine transmembrane segments (reviewed in [31]) which serves to couple the decarboxylation of carboxybiotin to the translocation of Na^+ from the cytoplasm to the periplasm. While there is no available X-ray crystal structure of this domain (nor, indeed, are there structural data for the complete OADC complex), the β-subunit has been subjected to extensive mutagenic analysis, with several key residues shown to be necessary for Na^+ binding, Na^+ channeling and biotin decarboxylation [31], [71], [72].

The small ~9 kDa γ-subunit is an integral membrane protein with a single membrane-spanning helix at the N-terminus, followed by a hydrophilic C-terminal domain which interacts with the α-subunit. The γ-subunit is essential for the overall stability of the complex, and likely serves as an anchor to hold the α- and β-subunits in place [73]. Furthermore, the γ-subunit significantly accelerates the rate of oxaloacetate decarboxylation in the α-subunit, and this correlates with the coordination of a Zn^{2+} metal ion by several residues at the hydrophilic C-terminus [73], [74]. It was originally proposed that the γ-subunit Zn^{2+} metal ion inserts into the carboxyltransferase domain active site of the α-subunit and serves as the Lewis acid metal center to stabilize the pyruvate enolate (the general carboxyltransferase domain mechanism is described in detail in a later section). However, the crystal structure of the carboxyltransferase domain from the α-subunit of *V. cholera* OADC revealed that the Zn^{2+} metal responsible for stabilization of the pyruvate enolate is bound within the carboxyltransferase domain itself and does not require the insertion of Zn^{2+} from the γ-subunit [67]. Instead, the site of interaction with the γ-subunit in *V. cholerae* OADC has been shown to be located in an intervening region between the carboxyltransferase and BCCP domains of the α-subunit, termed the “association domain” [75] (Fig. 5). This association domain resembles the allosteric/tetramerization domain of PC [68].

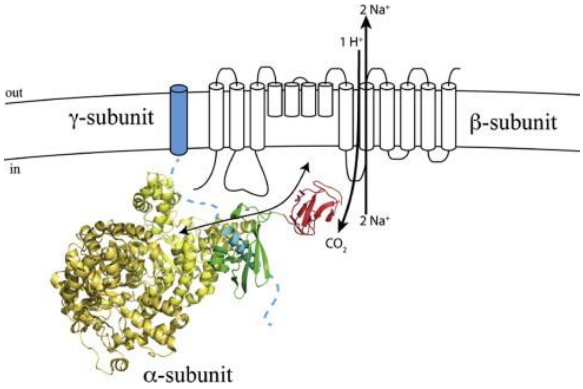

Fig. 5. A simplified model of the oxaloacetate decarboxylase complex structure. Oxaloacetate decarboxylase is composed of three separate subunits: the α-subunit (illustrated as a three-dimensional protein structure), the β-subunit (illustrated as a cartoon, colored in white) and the γ-subunit (illustrated as a cartoon, colored in blue). The γ-subunit is composed of a single membrane-spanning helix at the N-terminus (illustrated as a blue transmembrane cylinder) followed by a hydrophilic C-terminal domain (illustrated by dashed lines and a single α-helix) that interacts with the association domain of the α-subunit of monomer A (colored in green). The α-subunit is composed of an N-terminal carboxyltransferase domain dimer (monomer A is colored yellow;

monomer B is colored orange) and a C-terminal BCCP domain of monomer A (colored red). The arrows illustrate a hypothetical path traversed by the BCCP domain as it transits from the carboxyltransferase domain active site to the β-subunit. Carboxybiotin is decarboxylated in the β-subunit, resulting in the transport of Na^+ ions out of the cytosol. A more sophisticated model for BCCP translocation has recently been proposed, where the BCCP domain moves from the carboxyltransferase domain of one OADC complex to the β-subunit of a neighboring complex [68]. This illustration is based on the β-subunit topology determined by Dimroth (reviewed in [31]), the X-ray crystal structure of the carboxyltransferase domain of OADC (pdb i.d. = 2NX9; [67]) and the model for α/γ subunit interactions proposed by Balsera, Buey and Li [68]. The allosteric and BCCP domains are taken from the structure of *R. etli* PC (pdb i.d. = 2QF7) and are oriented according to a superimposition of the *R. etli* PC and OADC carboxyltransferase domains. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The overall reaction catalyzed by OADC couples the decarboxylation of oxaloacetate to the exchange of two intracellular Na^+ ions for one extracellular H^+ ion. The reaction is initiated by the enzyme-catalyzed decarboxylation of oxaloacetate in the carboxyltransferase domain of the α-subunit, yielding pyruvate and carboxybiotin. Subsequently, the C-terminal BCCP domain on the α-subunit translocates to the β-subunit where its decarboxylation is coupled to Na^+ translocation. As with PC, the OADC pump is reversible: at high concentrations of extracellular Na^+, the pump will couple the downhill movement of Na^+ into the cytosol with the carboxylation of pyruvate, to form oxaloacetate [76]. The biochemistry of OADC has been extensively studied and the mechanism of biotin decarboxylation and Na^+ translocation in the β-subunit has been comprehensively reviewed [31].

Transcarboxylase

Transcarboxylase (TC) plays a key role in the fermentation of malic and lactic acids by propionic acid bacteria, with propionic and acetic acids serving as the major end-products. Swick and Wood were the first to identify a biotin-dependent transcarboxylase activity in the cell-free extracts of the propionic-acid bacterium *P. shermanii* and showed that methylmalonyl CoA and pyruvate were the substrates for the reaction (Fig. 3; [77]). Harland Wood made monumental contributions to the study of biotin-dependent enzymes through his work on TC. Not long after his initial discovery of the enzyme, he purified the biotinylated enzyme [34] and, following several years of intense study, he showed that the enzyme was comprised of three distinct subunits: a 1.3S subunit that serves as the biotin carboxyl carrier protein, a 12S subunit that catalyzes the first half reaction (the decarboxylation of methylmalonyl-CoA to propionyl-CoA with formation of carboxybiotin) and a 5S subunit that catalyzes the second half reaction (the carboxylation of pyruvate to oxaloacetate) (Fig. 3; [78]). The complete enzyme consists of a hexameric central core of 12S subunits surrounded by six 5S dimers, each connected to the central core by twelve 1.3S biotin carrier subunits (reviewed in [79]). The structure is expected to be similar to the architecture observed in the recently reported holoenzyme structures of propionyl-CoA carboxylase and methylmalonyl-CoA carboxylase, where a central hexameric core is capped on each end by three α-subunits containing the carboxyltransferase and BCCP domains [80], [81]. In the forward direction, the overall reaction involves the conversion of methylmalonyl-CoA and pyruvate to propionyl-CoA and oxaloacetate. As was often emphasized by Wood in his many publications on the subject, TC is unique among the biotin-dependent enzymes in that CO_2 plays no role in the reaction, either as a substrate or a product.

More recently, Yee and colleagues reported the X-ray crystal structures of the 5S and 12S subunits providing, in the case of the 5S subunit, the first structure of a carboxyltransferase domain with biotin-dependent oxaloacetate decarboxylase activity [82], [83]. In a 2004 review, Carey, Sönnichsen and Yee elegantly summarized these structural advances in the context of Harland Wood's earlier pioneering work [84]. Since then, however, few studies on TC have been reported. Bhat and Berger showed by NMR that free biotin interacts with the carboxyltransferase domain of the 5S subunit and that biotin and pyruvate occupy different

binding sites [85], results that are consistent with X-ray crystal structures reported for the carboxyltransferase domain of PC [54].

The carboxyltransferase domains of TC and PC are functionally very similar. Both enzymes catalyze an identical reaction in the second partial reaction, namely the transfer of a carboxyl group from a covalently tethered carboxybiotin to pyruvate to generate oxaloacetate. While the TC reaction is typically drawn in the forward direction, the reaction is freely reversible and the carboxyltransferase domain is equally capable of catalyzing the decarboxylation of oxaloacetate (see, for example, [86]). As is the case with PC, therefore, it may also be considered a biotin-dependent oxaloacetate decarboxylase.

Carboxyltransferase domain structure

Recent X-ray crystal structures of PC [53], [54], [55], [62], the carboxyltransferase domain of the α-subunit of OADC (α-OADC) [67] and the 5S subunit of TC [83] have greatly advanced the description of catalysis in these enzymes. The carboxyltransferase domains from all three of these enzymes are homologous. The domain from *P. shermanii* 5S TC is 33% identical to human PC and the α-carbon atoms superimpose with a root-mean-square deviation of 1.4 Å. Similarly, the carboxyltransferase domain of α-OADC from *V. cholerae* is 34% identical to human PC and the α-carbon atoms correspond with a root-mean-square deviation of 1.3 Å. In all three cases, the carboxyltransferase domain crystallizes as a homodimer, in accordance with the dimerization state observed in solution. The active site architectures are virtually identical, suggesting that they share a common mechanism for stabilizing the pyruvate enolate.

The carboxyltransferase domain of the biotin-dependent oxaloacetate decarboxylases can be grouped into a larger family of TIM barrel metalloenzymes, called the “DRE-TIM” family, that catalyze carbon–carbon bond cleavage reactions proceeding through a stabilized enolate intermediate [87]. The overall domain architecture consists of two subdomains: an N-terminal canonical $(\beta/\alpha)_8$ TIM barrel fold and a large C-terminal extension, composed of nine α-helices (Fig. 6a). The C-terminal subdomain forms a funnel leading into the active site at the mouth of the $(\beta/\alpha)_8$ barrel. Structures of 5sTC, α-OADC, and PC all reveal a divalent metal cation bound in the active site (Zn^{2+}, Mn^{2+} or Co^{2+}), chelated by two conserved histidine residues (HxH) and a conserved aspartate (Fig. 6b). In some structures, but not all, the metal ion is further coordinated by the carboxylate oxygens of a carbamylated lysine residue [53], [54], [62], [83], [88]. A possible role for this carbamylated lysine in the catalytic mechanism is discussed below.

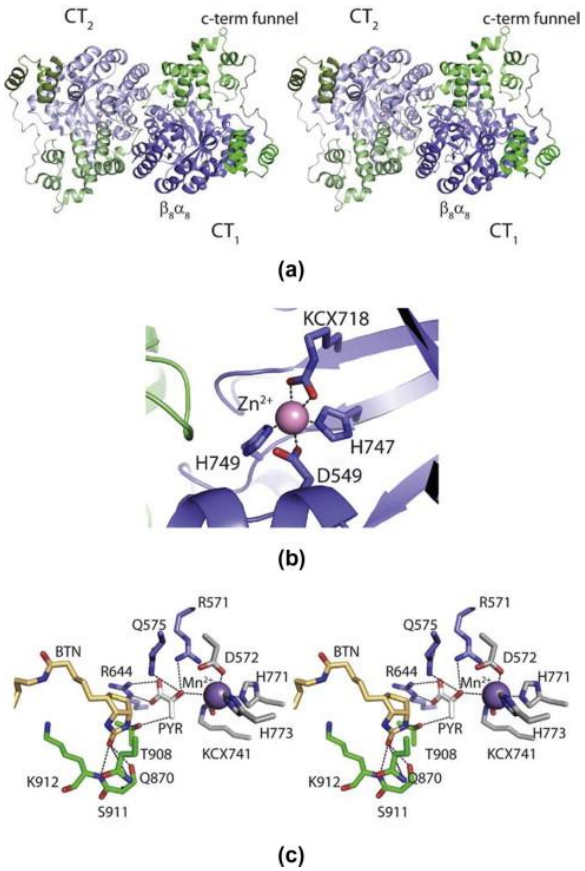

Fig. 6. The carboxyltransferase domain of pyruvate carboxylase. (a) A stereoview of a representative carboxyltransferase domain homodimer from *Rhizobium etli* PC (pdb i.d. = 2QF7). The carboxyltransferase domain is a homodimer consisting of a canonical $(\beta/\alpha)_8$ TIM barrel fold (blue) capped by a nine-helix C-terminal funnel (green). (b) The carboxytransferase active site centers on a divalent cation coordinated by a conserved pair of His residues (HxH) and a conserved Asp, with a carbamylated Lys (labeled KCX) coordinating the metal ion in some structures, as shown for the active site of *R. etli* PC (pdb i.d. = 2QF7). (c) A stereoview highlighting substrates and important residues in the carboxyltransferase active site of human PC. Tethered biotin (BTN; orange) is bound in the active site of human PC (pdb id = 3BG3), with the N-1 nitrogen positioned 4.5 Å from the β-carbon of pyruvate (PYR). A conserved Thr residue (T908 in human PC) serves to shuttle a proton between biotin and pyruvate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In PC enzymes, the identity of the tightly bound metal ion is species-specific. Vertebrates predominantly bind Mn^{2+}, whereas yeast and bacteria prefer Zn^{2+} [37]. On the other hand, the 5S subunit of TC primarily binds Co^{2+} in its active site [83], but there is also evidence for Zn^{2+} binding [89], [90]. α-OADC purified from source binds Zn^{2+} exclusively, but can also bind Co^{2+} when heterologously expressed in *E. coli* [67]. It is clear that the metal ion in the carboxyltransferase domain plays an important functional role in all three enzymes. Mutating the metal-chelating aspartate in PC from *Bacillus thermodenitrificans* results in a 22-fold reduction in pyruvate carboxylation activity and a 54-fold increase in K_M for pyruvate [91]. In the carboxyltransferase domain of OADC from *V. cholerae*, mutations in either the aspartate or the two histidine residues that coordinate the active site metal results in a complete loss of enzyme activity. Mutating the conserved metal-chelating lysine either substantially reduces or completely eliminates enzyme activity in all three enzymes [53], [61], [67], [83], [91]. In addition, the physical removal of the tightly bound metal in TC [89], PC [92] and OADC [93] causes aggregation and enzyme dissociation, suggesting that the metal ion plays a critical role in maintaining the structural integrity of the enzyme.

Oxaloacetate decarboxylation mechanism

Well before the structure of the carboxyltransferase domain was known, three central mechanistic features were established through studies conducted on both PC and TC: (1) the carboxylation of pyruvate to oxaloacetate proceeds with retention of configuration at the carbon atom undergoing carboxylation [94], [95]; (2) the reaction proceeds by a stepwise mechanism [96], [97]; (3) in PC, carboxybiotin does not translocate to the carboxyltransferase domain before pyruvate or a pyruvate analog binds in the active site [98], [99], [100]. While major structural advances in recent years have considerably advanced the description of catalysis in the carboxyltransferase domain, the mechanistic details have remained consistent with these earlier observations. The biotin-dependent decarboxylation reaction in the carboxyltransferase domain of PC, OADC and TC follows a stepwise mechanism that proceeds through both a stabilized enolpyruvate intermediate and a biotin enolate intermediate (Fig. 7). Since most of the recent advances in describing the chemical mechanism of the carboxyltransferase domain have come from studies on PC, this enzyme will be highlighted. However, wherever possible, the conclusions are extended to the broader context of all three biotin-dependent oxaloacetate decarboxylase enzymes.

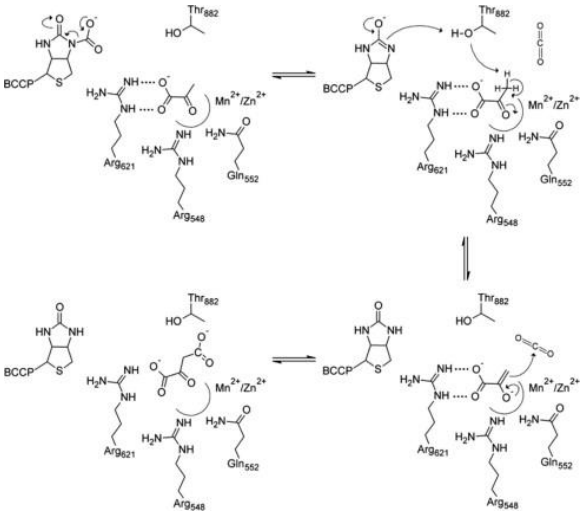

Fig. 7. The pyruvate carboxylation mechanism for the carboxyltransferase domain of *R. etli* pyruvate carboxylase. This research was originally published in The Journal of Biological Chemistry. Adam Lietzan and Martin St. Maurice. A Substrate-induced Biotin Binding Pocket in the carboxyltransferase Domain of Pyruvate Carboxylase. J. Biol. Chem. 2013; 288: 19,915–19,925.

Stabilization of the enolpyruvate intermediate

Prior to determination of the X-ray structures of the carboxyltransferase domain, it was well established that a tightly bound divalent metal ion was essential for activity in TC, OADC and PC and that the pyruvate binding site in PC was located near this metal ion [89], [93], [101], [102], [103]. Since then, several structures have revealed substrates or substrate analogs bound near the active site metal ion in the carboxyltransferase domain of both PC and TC [54], [62], [83], [104]. Given the proximity of substrates and substrate analogs to the divalent cation, it has been proposed that the metal ion serves to stabilize the enolpyruvate intermediate that is generated following the decarboxylation of oxaloacetate [54], [61], [67]. However, while the metal ion is often illustrated in reaction schemes as forming a bidentate interaction with the β-keto acid substrate, there is, as yet, no structural evidence to support a direct interaction between the substrate and the metal ion [104].

Yee and coworkers were the first to suggest that the metal ion does not directly participate in stabilizing the pyruvate enolate but serves, instead, to bind and orient the substrate [83]. Many substrate-bound

carboxyltransferase domain structures have since been reported for PC and, in all cases, the carbonyl oxygen of substrate and substrate/product analogs resides at a greater distance (>3.5 Å) from the metal ion than would be expected from direct chelation [54], [62], [104]. In all carboxyltransferase domain structures, a conserved glutamine/arginine pair (RXXXQ; Arg_{571}/Gln_{575} in human PC) is positioned in close proximity to the carbonyl oxygen of the substrate (Fig. 6c). These residues may provide sufficient stabilization to the enolpyruvate intermediate that a direct interaction with the metal ion is unnecessary for catalysis. Notably, the glutamine/arginine pair (Arg_{571}/Gln_{575} in human PC) is conserved in the broader DRE-TIM metalloenzyme family that catalyzes carbon–carbon bond cleavage on substrates that proceed through stabilization of an enolate intermediate [87]. Mutation of the Arg/Gln pair in *R. etli* PC (Arg_{548} and Gln_{552}) results in substantially reduced stabilization of the enolate intermediate without significantly perturbing substrate binding [105]. Furthermore, mutating the analogous arginine and glutamine residues in OADC completely eliminates enzyme activity [67]. The structures of *R. etli, S. aureus*, and human PC with bound substrate and substrate/product analogs identified an additional conserved active site arginine (Arg_{644} in human PC; Fig. 6c) that forms a salt bridge with the carboxylate group of pyruvate [54], [62], [104]. Mutating the equivalent arginine in *S. aureus* PC eliminates pyruvate carboxylation activity [55]. This residue is only conserved within the homologous carboxyltransferase domains from the biotin-dependent oxaloacetate decarboxylases and not in the broader DRE-TIM family, suggesting that it is specifically positioned to interact with and orient the pyruvate/oxaloacetate substrate.

Structures of the carboxyltransferase domain from several PC enzymes, OADC and TC have revealed substantial variation in the degree of carbamylation of a conserved lysine residue positioned within the coordination sphere of the active site metal ion (see KCX in Fig. 6b and c). This residue appears to primarily contribute to coordination of the metal ion, since it is always observed interacting with the metal ion regardless of its carbamylation state in all PC enzyme structures. However, in the structure of *P. shermanii* 5S TC, the lysine side chain is carbamylated in the apo enzyme, is partially carbamylated in the pyruvate bound state, and is not carbamylated when oxaloacetate is bound [83]. These observations led Hall et al. and Studer et al. to propose that the carbamylated lysine serves as a carboxylate carrier between oxaloacetate and carboxybiotin [67], [83]. Mutation of the carbamylated lysine in both 5S TC and α-OADC leads to a complete loss of enzyme activity, consistent with this hypothesis [67], [83]. More recent structural and kinetic data from PC, however, raise questions about the universality of this proposed mechanism. For example, mutations of this lysine residue in *B. thermodenitrificans* PC [91] and *R. etli* PC [53], [61] maintain 1–5% of wild type activity, indicating that, while this residue is important, it is not essential for PC catalysis. Furthermore, the structures of *S. aureus* and human PC with bound BCCP-biotin reveal that 1'-N of biotin is located ~8 Å from the carbamylated lysine residue (Fig. 6c), precluding any possibility of direct transfer. Since there is, as yet, no structure of 5S TC or α-OADC with biotin bound in the active site, it is unclear whether these homologous enzymes use different mechanisms for carboxylate transfer or whether the role of the carbamylated lysine residue in 5S TC and α-OADC has been misinterpreted.

Carboxyl group transfer to biotin and stabilization of the biotin enolate intermediate

The role of biotin in the pyruvate/oxaloacetate carboxyltransfer reaction has been greatly informed by the structures of the *S. aureus* and human PC that revealed a tethered biotin bound in the carboxyltransferase domain active site ~5 Å removed from the β-carbon of pyruvate, a distance too great for direct proton/carboxyl transfer between substrates [54]. The ureido oxygen of biotin is positioned in close proximity to a conserved serine side chain and adjacent backbone amide group (e.g. Ser_{911} and Lys_{912} in human PC; Fig. 6c), located in the C-terminal subdomain funnel of all biotin-dependent oxaloacetate decarboxylases. In addition, a glutamine side chain from helix 10 (Gln_{870} in human PC; Fig. 6c) projects toward the ureido oxygen of biotin in PC, but this residue is not conserved in α-OADC or 5S TC. Interestingly, helix 10 represents a rare case of modest structural deviation between PC, OADC and TC: this helix originates from deeper within the active site cavity in

PC than do the equivalent helices in OADC or 5S TC. It has been proposed that the serine and glutamine side-chains, together with the backbone amide nitrogen, contribute to a tetrahedral geometry near the biotin ureido oxygen that likely serves to facilitate the decarboxylation of biotin by stabilizing the negative charge on the sp^3 oxyanion of the biotin enolate intermediate [54], [61]. In the existing PC crystal structures, the ureido oxygen of biotin is located near this tetrahedral coordination sphere but is not centered within it. However, the bulkier carboxybiotin substrate is likely to occupy a slightly altered position in the binding site that may result in centering the ureido oxygen for maximum stabilization. While these residues have not been subjected to mutagenesis in TC or OADC, mutation of the glutamine and serine side chains in PC leads to a reduced rate of oxaloacetate decarboxylation, which is proposed to result from a weakened stabilization of the biotin enolate in the rate-limiting biotin decarboxylation step [61].

The carboxyltransfer reaction between oxaloacetate and biotin has been most extensively studied in PC. A pH rate profile of the oxaloacetate decarboxylation reaction revealed no ionizable groups, leading Attwood and Cleland to propose that direct proton transfer occurs between pyruvate and biotin, and that this step follows the initial decarboxylation of carboxybiotin (forward) or oxaloacetate (reverse) [60]. Deuterium isotope and ^{13}C isotope effects have also been observed in PC, indicating that both proton and carboxylate transfer are partially rate limiting and that there are separate proton and carboxylate transfer steps in the reaction [96], [106]. Stepwise transfer has also been reported in TC [97]. Most recently, structural and site-directed mutagenic studies have unveiled a critical role for a conserved threonine side chain, positioned between the N-1' of biotin and the β-carbon of pyruvate in PC and ideally poised to bridge the ~5 Å gap between these two substrates (Fig. 6c). This threonine side chain, conserved in all three biotin-dependent oxaloacetate decarboxylase enzymes, shuttles a proton between the enolates of biotin and pyruvate and is indispensable for catalysis (Fig. 7) [54], [61]. The threonine side chain rests at the base of the C-terminal extension funnel of the carboxyltransferase domain. While the threonine residue responsible for proton shuttling is present in all three enzymes, to our knowledge, this residue has not yet been subjected to mutagenic analysis in either TC or OADC. We predict that such studies would confirm a common proton shuttling mechanism associated with carboxyl transfer in the biotin-dependent oxaloacetate decarboxylases and would also confirm that a common biotin binding site is present in all three enzymes. Consistent with this prediction, Bhat and Berger recently showed that biotin and pyruvate occupy different binding sites in TC [85], suggesting that biotin and pyruvate are separated in TC just as they are in PC.

The most recent description of the chemical mechanism for carboxyl group transfer between oxaloacetate and biotin is summarized using the specific example of *R. etli* PC in Fig. 7. In PC and TC, this reaction is typically drawn proceeding in the direction of pyruvate carboxylation. The reaction is freely reversible, however, and proceeds in the opposite direction for oxaloacetate decarboxylation. Carboxybiotin, tethered to BCCP, enters the active site after pyruvate binds (described in greater detail below). Once bound, carboxybiotin decarboxylation is promoted through biotin enolate stabilization in the conserved active site triad and, potentially, through a proposed rotation of the carboxyl group out of plane with the ureido ring [107]. A proton is subsequently shuttled from pyruvate to the biotin enolate via the conserved threonine residue, forming an enolpyruvate intermediate. Finally, the previously liberated CO_2 is transferred to pyruvate, generating oxaloacetate. The stabilization of the enolpyruvate intermediate by conserved active site residues and possibly the tightly bound metal ion serves to increase the acidity of the methyl group protons and facilitate proton abstraction by the conserved threonine.

It is noteworthy that nine disease-causing single nucleotide substitutions have been identified in human PC [108]. Of these, four (R583L, A610T, R631Q and M743I) have been mapped to the carboxyltransferase domain and the residues are conserved in all three biotin-dependent oxaloacetate decarboxylase enzymes. The A610T mutation is located at the opening of the active site. The bulkier threonine partially precludes the binding

of biotin in the active site, thus greatly reducing enzyme activity [55], [83]. The M743I is located deep in the active site where it most likely disrupts pyruvate binding [54], [83]. The R583L and R631Q mutations are located at the surface of the CT domain, distant from the active site. It has been proposed that these mutations may induce a conformational change that alters the interaction between domains and interferes with the transmission of the regulatory signal from acetyl-CoA [108].

Interaction between the CT and BCCP domains

Whereas the detailed chemical mechanism for biotin-dependent carboxyl transfer now appears well-resolved, there are many remaining questions surrounding the interaction between the BCCP and carboxyltransferase domains and how these inter-domain interactions contribute, if at all, to the coordination of catalysis that exists between physically separate active sites. Structural insights in this regard are limited to PC, since X-ray crystal structures have not yet been reported for the combined BCCP and carboxyltransferase domains in either TC or OADC.

The *S. aureus* PC and human PC structures afforded the first snapshots of the BCCP-carboxyltransferase domain interaction [54]. In these structures, the BCCP domain is captured binding in the C-terminal funnel of the carboxyltransferase domain with the biotinylated MKME hairpin extending deeply into the funnel, allowing biotin to reach into the active site (Fig. 6c). In response to the docking of the BCCP domain, several α-helices (most notably, helix 10) in the C-terminal funnel shift inwards to create a more “closed” conformation. This may allow the binding of BCCP to transmit an effect into the active site through the changes in the relative positioning of the tethered biotin, the threonine, and the pyruvate/oxaloacetate substrate. Aside from this general set of movements, however, very few specific side chain interactions are observed between BCCP and CT [54]. This lack of specific interactions is not surprising given that the BCCP domain must freely translocate between individual catalytic domains. A similar paucity of specific inter-domain interactions with BCCP has been repeatedly observed in the holoenzyme structures of other biotin-dependent enzymes [80], [81], [88], [109].

The dependence of oxaloacetate decarboxylation on BCCP-biotin remains to be established. While it appears that the 5S TC subunit is unable to catalyze oxaloacetate decarboxylation in the absence of an intact BCCP domain [110], there are several reports in the PC literature suggesting that the enzyme can catalyze oxaloacetate decarboxylation in the absence of BCCP-biotin, albeit at a greatly reduced rate [60], [62], [105], [111], [112]. To our knowledge, similar studies have not yet been performed in α-OADC. It is certainly possible to envision oxaloacetate decarboxylation proceeding in a biotin-independent manner, in a reaction similar to what has been proposed for the non-homologous oxaloacetate decarboxylase PA4872 from *Pseudomonas putida*, where a water molecule is thought to serve as the acid that donates a proton to the pyruvate enolate [113]. The crystal structure of the carboxyltransferase domain from *R. etli* PC with pyruvate (pdb i.d. 4JX5) contains several ordered water molecules in the active site [62]. Furthermore, the pK_a of water (15.7) is less than the pK_a for the N-1 of biotin (~17 [11]), making it an equivalent proton donor, provided that the hydrogen-bonding network in the active site can stabilize the hydroxide ion in a similar manner to the biotin enolate. The stabilized hydroxide will subsequently react with the liberated CO_2 to form bicarbonate, completing the reaction cycle. Further studies are warranted to determine the degree to which both biotin and BCCP contribute to the catalytic rate in the carboxyltransferase domain of these enzymes.

A major unanswered question surrounding the biotin-dependent enzymes is centered on the mechanism responsible for the coordination and control of BCCP access to the individual active sites during catalytic turnover. For example, Goodall et al. showed for sheep liver PC that N-[^{14}C]-carboxybiotin is quite stable in the absence of substrates, but that several carboxyltransferase domain substrates and competitive inhibitors of pyruvate are sufficient to invoke the translocation and decarboxylation of N-[^{14}C]-carboxybiotin in the carboxyltransferase domain [98]. These experiments demonstrated that carboxybiotin rapidly decarboxylates in

the carboxyltransferase domain, even in the absence of the bona fide acceptor substrate, and that a carboxyltransferase domain ligand is required to facilitate BCCP-biotin access to the active site. Recently, we described a substrate-induced biotin binding pocket in the carboxyltransferase domain of *R. etli* PC that provides a structural explanation for the phenomenon described by Goodall et al. [62]. Structures of the *R. etli* carboxyltransferase domain, in the presence and absence of substrates and substrate analogs, reveal that the biotin binding site in the carboxyltransferase domain forms as a consequence of pyruvate binding in the active site. The active site is reconfigured upon pyruvate binding, from an "open" conformation to a "closed" conformation (Fig. 8). This conformational rearrangement is initiated by the interaction of the substrate carboxylate with the conserved active site arginine mentioned previously (Arg_{644} in human PC; Fig. 6c) and is stabilized by the interaction of a conserved aspartate and tyrosine residue (e.g. Asp_{590} and Tyr_{628} in *R. etli* PC), resulting in the formation of the biotin-binding pocket. Both the tyrosine and aspartate residues are conserved in OADC and TC, suggesting that a common mechanism extends across all of the biotin-dependent oxaloacetate decarboxylase enzymes. Interestingly, the X-ray crystal structure of the carboxyltransferase domain from *V. cholera* OADC also displays an open conformation in the absence of bound substrate [67] and both fluorescence and infrared data demonstrate that ligand binding restricts the tryptophan microenvironment and promotes structural changes in the carboxyltransferase domain of OADC [114] consistent with what is observed in the structures of PC.

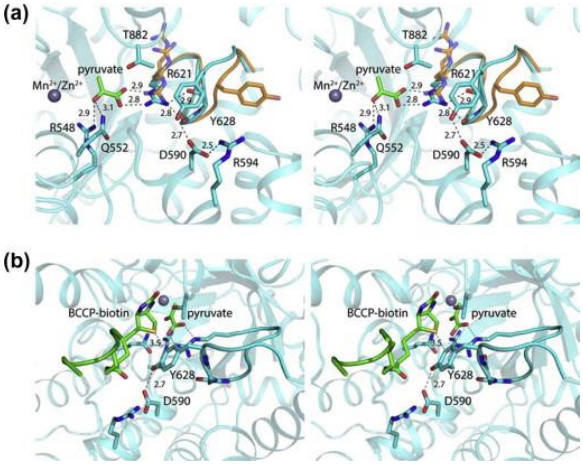

Fig. 8. Substrate binding leads to active site remodeling in the carboxyltransferase (CT) domain of *Re*PC. (a). Stereo view of the active site from the carboxyltransferase domain upon pyruvate binding, showing the change from the open conformation (orange) to the closed conformation (cyan) following substrate 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 *Re*PC wild-type (pdb i.d. 2QF7). (b**)** Structural alignment of the pyruvate-bound ΔBCΔBCCP *Re*PC with the *Hs*PC crystal structure (pdb i.d. 3BG3; monomer B; r.m.s. deviation = 1.3 Å) reveals the expected positioning of biotin in the carboxyltransferase domain active site of *Re*PC. In the closed conformation, Asp_{590} and Tyr_{628} form a surface to assist with biotin insertion into the active site. This research was originally published in The Journal of Biological Chemistry. Adam Lietzan and Martin St. Maurice. A Substrate-induced Biotin Binding Pocket in the carboxyltransferase domain of pyruvate carboxylase. J. Biol. Chem. 2013; 288: 19,915–19,925.

Allosteric/tetramerization domain of PC and the association domain of OADC

While not directly part of the carboxyltransferase domain, a final section of this review is devoted to the similarities in a small structural domain that neighbors the carboxyltransferase domain in both OADC and PC. Most α_4 forms of PC are subject to regulation by the non-essential, allosteric activator acetyl-coenzyme A, which

enhances pyruvate carboxylation activity and stabilizes the tetramer (reviewed in [115]). The holoenzyme structures of α_4 PC from *R. etli* and *S. aureus*, along with the structure of the biotin carboxylase domain truncation of α_4 human PC, revealed a structural domain linking the biotin carboxylase, CT and BCCP domains (Fig. 4, green) [53], [54]. This small domain, termed the "allosteric" domain in *R. etli* PC and the "PC tetramerization" domain in *S. aureus* and human PC, consists of a central helix that connects the biotin carboxylase and carboxyltransferase domains. It is surrounded by a highly-twisted, four-stranded anti-parallel β-sheet that links the C-terminus of the carboxyltransferase domain to the BCCP domain.

This domain appears to play multiple roles in PC. Most notably, it serves as the binding site for the nucleotide moiety of the allosteric activator, acetyl-CoA [53], [55]. A mutation in Arg451 (R451C), located in close proximity to the acetyl-CoA binding site, is associated with human PC deficiency. This R451C mutation was kinetically characterized and was shown to significantly reduce the sensitivity of PC to acetyl-CoA activation [54]. In addition to serving as the acetyl-CoA binding site, the allosteric domain in *S. aureus* and human PC is necessary for tetramerization, forming homodimer contacts across the face of the tetramer [54], [55]. Finally, structures of *S. aureus* and *R. etli* pyruvate carboxylases have revealed a biotin binding pocket, termed the exo-binding site, at the interface between the allosteric domain and the carboxyltransferase domain [41], [54]. This exo-binding site was not observed in the structure of human PC, where a conformational change in a C-terminal loop precluded access to the site [54]. It is not known what role, if any, the exo-binding site plays in the overall reaction catalyzed by PC.

Balsera et al. [68] recently reported the stoichiometry and SAXS-based structure of the soluble α-subunit and the cytosolic portion of the γ-subunit (termed γ') from *V. cholera* OADC. Their results suggest that tetramerization of α-OADC is mediated by an interaction between the "association domain" of the α-subunit and γ'-subunit in a manner that is completely analogous to the allosteric domain of PC. In an intriguing model, it was proposed that BCCP-biotin is carboxylated in the carboxyltransferase domain of a neighboring α-OADC. The interaction is facilitated by the tetramerization of α-OADC through interactions between the association domain and the γ'-subunit, which maintain two of the four α-OADC molecules in close proximity to the membrane-bound β-subunit. This proposal is consistent with the observed subunit stoichiometry and invokes a half-the-sites-reactivity model similar to what has been suggested for other biotin-dependent enzymes. Most interestingly, the γ'-subunit appears to stabilize a four-stranded β-sandwich in the association domain of the α-subunit that is proposed to act analogously to the central α-helix of the allosteric domain in PC.

It is tempting to use the observations in OADC and PC to speculate about inter-subunit association in TC, where the ~50 amino acid N-terminal tail of the 1.3S subunit is required for attachment to the 5S and 12S subunits [116]. The structure of the N-terminus of the isolated 1.3S subunit was highly disordered and could not be determined by NMR [117]. Given that elements of the PC allosteric domain have now been observed in PC, OADC, propionyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase, it is conceivable that the disordered N-terminal domain of the 1.3S subunit takes on a fold similar to the allosteric domain of PC and associates with neighboring subunits in a manner analogous to the γ-/association domain interaction of OADC.

Summary

Nearly a decade ago, Yee and colleagues reported the first structures of the carboxyltransferase domain from the 5S subunit of transcarboxylase. This marked the first of many major structural advances in the biotin-dependent enzyme family that followed, a few years later, with the structures of both oxaloacetate decarboxylase and pyruvate carboxylase. This wealth of structural information has led to detailed mutagenic studies in the carboxyltransferase domains of all three biotin-dependent oxaloacetate decarboxylases and has revealed what appear to be many common mechanistic features across all three enzymes. Nevertheless, many questions remain unresolved, particularly with respect to the mechanism of carboxyl group transfer

between biotin and oxaloacetate. The role of the carbamylated lysine in the carboxyl transfer reaction remains ambiguous and there are insufficient studies to determine whether these three enzymes maintain a common set of interactions between the biotin carrier protein and the carboxyltransferase domain. The conservation in structure and mechanism serves as a reminder that studies conducted in one enzyme should serve to prompt parallel studies in the homologous enzymes. As studies of these enzymes continue to emerge, it will become increasingly possible to delineate the similarities and differences within this subset of biotin-dependent enzymes, and from this, to extend these findings to more general conclusions about the biotin-dependent enzyme family as a whole.

Acknowledgment

We thank Dr. Patrick Frantom for drawing our attention to the placement of the biotin-dependent oxaloacetate decarboxylase enzymes within the larger DRE-TIM metalloenzyme family.

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[1]*Abbrevation used:* CoA, acetyl coenzyme A; BCCP, biotin carboxyl carrier protein; BC, biotin carboxylase; ODC, oxaloacetate decarboxylase; ACP, acyl carrier protein; PC, pyruvate carboxylase; OADC, oxaloacetate decarboxylase; TC, transcarboxylase; TCA, tricarboxylic acid; α-OADC, α-subunit of OADC.