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The Enzymes of Biotin Dependent CO₂ Metabolism: What Structures Reveal About Their Reaction Mechanisms

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Abstract

Biotin is the major cofactor involved in carbon dioxide metabolism. Indeed, biotin-dependent enzymes are ubiquitous in nature and are involved in a myriad of metabolic processes including fatty acid synthesis and gluconeogenesis. The cofactor, itself, is composed of a ureido ring, a tetrahydrothiophene ring, and a valeric acid

side chain. It is the ureido ring that functions as the CO₂ carrier. A complete understanding of biotin-dependent enzymes is critically important for translational research in light of the fact that some of these enzymes serve as targets for anti-obesity agents, antibiotics, and herbicides. Prior to 1990, however, there was a dearth of information regarding the molecular architectures of biotin-dependent enzymes. In recent years there has been an explosion in the number of three-dimensional structures reported for these proteins. Here we review our current understanding of the structures and functions of biotin-dependent enzymes. In addition, we provide a critical analysis of what these structures have and have not revealed about biotin-dependent catalysis.

Introduction

As with many scientific advances, the discovery of biotin (also known as vitamin H or vitamin B₇) happened from a seemingly simply observation; raw egg whites were known to be toxic to humans, and this toxicity could be prevented by an unknown substance found in egg yolks. In 1936, Kögl and Tönnis reported the discovery and isolation of the egg yolk factor, which ultimately turned out to be biotin.¹ Why then are raw egg whites toxic? They contain the protein avidin, which essentially binds biotin irreversibly thereby preventing absorption.² The structure of biotin [Fig. 1(a)] was subsequently determined in 1942 by Du Vigneaud,³ and in the late 1940s its role in metabolism was unraveled through a series of elegant studies by Henry Lardy and colleagues who found that it was required for carboxylation reactions.⁴ Ten years later the pioneering efforts of Salih Wakil demonstrated that biotin was covalently attached to acetyl-CoA carboxylase thus proving it to be an essential cofactor for enzyme catalysis.⁵ It is now well established that the primary biological role of biotin is to serve as an enzymic cofactor required for the transfer of carbon dioxide.

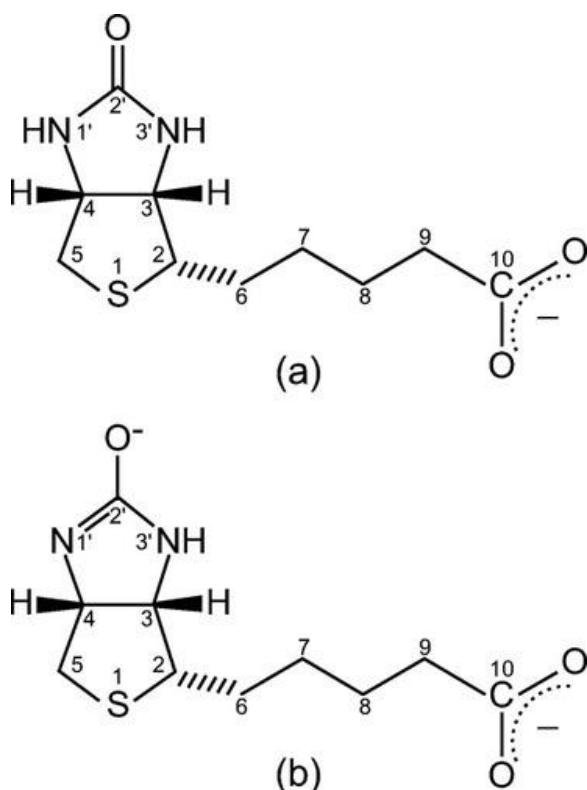
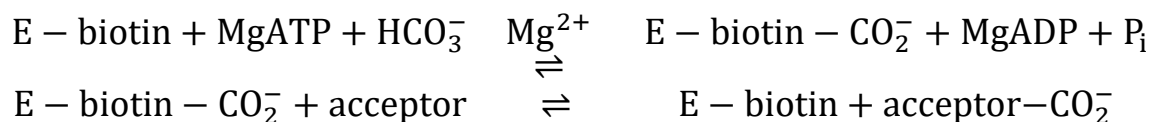


Figure 1 Structure of biotin. The enolate form of the cofactor is shown in (b).

Catalysis by all biotin-dependent enzymes involves two half-reactions. Biotin-dependent enzymes can be divided into three different classes depending upon whether they function as carboxylases (Class I), decarboxylases (Class II), or transcarboxylases (Class III). In most cases, biotin-dependent enzymes catalyze the fixation of CO₂ and thus belong to the Class I family.

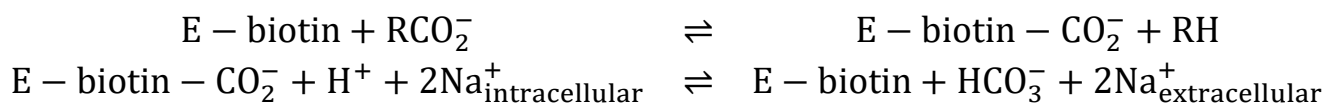
The general reactions catalyzed by the Class I enzymes are shown in Scheme 1. Their catalytic mechanisms are known to proceed through carboxyphosphate intermediates. It is still not entirely clear as to whether carboxyphosphate decomposes into CO_2 and P_i followed by reaction of CO_2 with biotin or whether the carboxylate group is transferred directly from carboxyphosphate to biotin. One intriguing proposal suggests that the first half reaction involves substrate-assisted catalysis whereby P_i serves as the general base required to abstract the N1' proton from biotin to generate a biotin "enolate-like" intermediate [Fig. 1(b)].⁶⁻⁸ Indeed, the nucleophilicity of biotin would be greatly increased by such an abstraction event.



Scheme 1

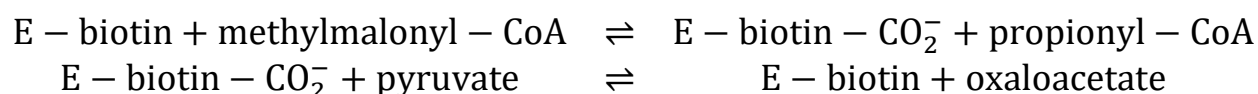
As shown in Scheme 1, the first half reaction for a Class I enzyme requires two metal ions, usually magnesium, for activity. One metal ion is chelated to ATP such that the metal-nucleotide complex is the actual substrate. The second metal ion binds in the active site separately from the other substrates and because its concentration does not change over the course of the reaction, it is considered a pseudosubstrate. In the second half-reaction, the carboxylate group attached to biotin is transferred to an acceptor molecule residing in a completely separate active site. The identity of the acceptor gives rise to the name of the enzyme. For example, if pyruvate is the acceptor then the enzyme is pyruvate carboxylase. Eukaryotic biotin-dependent enzymes only catalyze carboxylation reactions. In mammals, the biotin-dependent enzymes and the metabolic processes they participate in are as follows: pyruvate carboxylase in gluconeogenesis; propionyl-CoA carboxylase in odd-chain fatty acid oxidation; acetyl-CoA carboxylase in fatty acid synthesis; and β -methylcrotonyl-CoA carboxylase in amino acid catabolism. There are two additional biotin-dependent carboxylases not found in eukaryotes: urea amidolyase, which enables microorganisms to use urea as a nitrogen source and geranyl-CoA carboxylase, which is involved in isoprenoid catabolism in microorganisms and plants.

The two half-reactions catalyzed by the Class II decarboxylases are outlined in Scheme 2. Unlike the carboxylases, CO_2 is not transferred to an acceptor molecule, but instead is released as bicarbonate. There are three sources of CO_2 (designated R in Scheme 2) that can carboxylate biotin and which correspond to the name of the enzyme: oxaloacetate decarboxylase, methylmalonyl-CoA decarboxylase, and glutamyl-CoA decarboxylase. These biotin-dependent decarboxylases are found in anaerobic bacteria where they function in the pumping of sodium ions against a concentration gradient without the use of ATP.



Scheme 2

Thus far, there is only one Class III family member that has been identified, methylmalonyl CoA-oxaloacetate transcarboxylase. It is involved in propionic acid production in the bacterium *Propionibacterium shermanii*,⁹ and it is quite unique in that it does not use free CO_2 . In addition, neither ATP nor a divalent metal ion is required. The two half-reactions for this enzyme are shown in Scheme 3.



Scheme 3

In all three of the reactions outlined in Schemes **1**, **2**, and **3**, biotin is designated as E-biotin to indicate that the cofactor is covalently attached to the enzyme. The attachment is made via an amide linkage between the carboxylate group of the biotin valerate side chain [Fig. **1(a)**] and the ϵ -amino group of a specific lysine residue. The formation of the amide bond is a posttranslational modification event that is catalyzed by holocarboxylase synthase (also known as biotin protein ligase). The specific lysine residue that is modified is contained within the tetrapeptide sequence Ala-Met-Lys-Met. This sequence is not absolutely invariant, however, because in some of the urea amidolyases the lysine is contained within the following sequences: Ser-Met-Lys-Met or Ala-Met-Lys-Ala or Ala-Met-Lys-Thr.¹⁰

At present there are more than 70 X-ray coordinate files deposited in the Protein Data Bank for structures of biotin-dependent enzymes. An elegant review focusing on the holoenzyme structures of various biotin-dependent carboxylases has recently been published.¹¹ Due to space limitations, however, details concerning the chemical mechanisms of the individual components were necessarily lacking. This article serves to fill the gap by describing the structural and functional aspects of the individual components. For a description of the entire holoenzyme complexes, please see Tong, 2012.¹¹

Overall Structure of a Biotin Carboxylation Domain: The First Half-Reaction

Acetyl-CoA carboxylase, a Class I family member, catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA. This reaction constitutes the first committed step in the biosynthesis of long chain fatty acids.¹² In *Escherichia coli*, the enzyme is composed of three subunits that are isolated separately and that display distinct functional properties.¹³ The biotin carboxylase subunit catalyzes the first half reaction (Scheme **1**), whereas the carboxyltransferase subunit carries out the second half reaction. The third component, the biotin carboxyl carrier protein (BCCP), contains the covalently attached biotin cofactor. BCCP functions as a “swinging arm” that allows the cofactor to shuttle between the biotin carboxylase and carboxyltransferase subunits of the complex.

The structure of biotin carboxylase from *E. coli* was reported in 1994, and it represented the first glimpse of a biotin-dependent carboxylase.¹⁴ A ribbon representation of one subunit of the dimeric enzyme is shown in Figure **2(a)**. In this initial structural analysis, a phosphate ion was observed binding in the active site cleft of the enzyme. As can be seen, the subunit is somewhat asymmetric with a small domain extending away from the main body of the molecule. Overall the architecture of the enzyme can be envisioned in terms of three domains. The A-domain consists of a five-stranded parallel β -sheet flanked on either side by a total of four α -helices. Following this N-terminal region is the B-domain, which contains a three-stranded anti-parallel β -sheet covered on one side by two α -helices. The C-domain is the most complicated of the three regions. It contains an eight-stranded antiparallel β -sheet, a three-stranded anti-parallel β -sheet, and seven α -helices. The A- and B-domains are connected by a helix-turn-helix motif with the two helices oriented at approximately 90° with respect to one another. These helices are bridged by Asp 115 which adopts ϕ, ψ angles of -119° and 122°, and this helix-turn-helix motif is a hallmark of proteins belonging to the ATP grasp superfamily as discussed below.

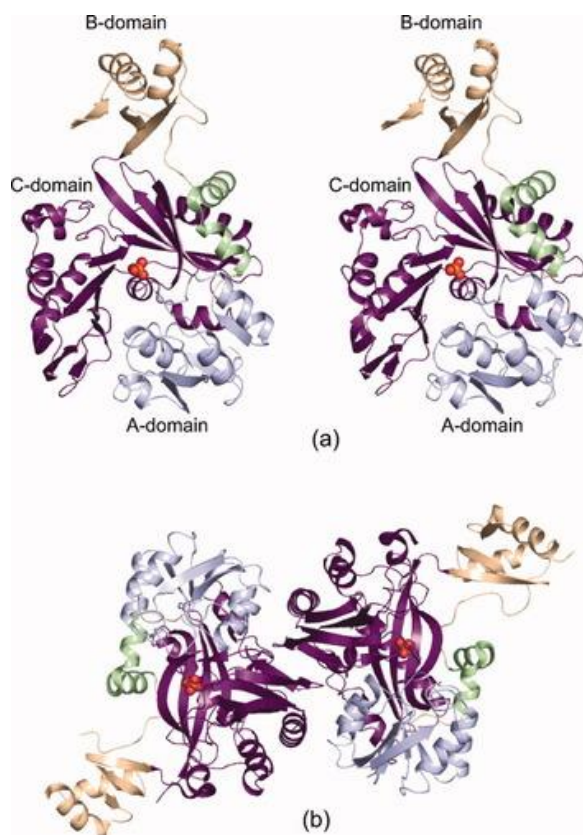


Figure 2 Structure of the *E. coli* biotin carboxylase. A stereo-view of one subunit of the enzyme is shown in (a). The A-, B-, and C-domains are displayed in blue, wheat, and violet, respectively, and the bound phosphate ion is shown in a sphere representation. The quaternary structure of the enzyme is depicted in (b). The two-fold rotational axis relating one subunit to another is nearly perpendicular to the plane of the page. X-ray coordinates were obtained from the Protein Data Bank (**1BNC**). All structure figures were prepared with the software package PyMOL.⁹⁴

Shown in Figure 2(b) is a ribbon representation of the biotin carboxylase dimer. The B-domains for each subunit are located on opposite edges of the dimer, nearly 90 Å from one another. The subunit–subunit interface is formed by three α -helices (two from the A- and one from the C-domain) and a β -strand-turn- β -strand motif delineated by Phe 357 to Tyr 372. This strand-turn-strand secondary structural element serves to bridge the eight-stranded β -sheets in each C-domain, thereby forming an approximate 16-stranded antiparallel β -sheet across the dimer interface. Given the evidence that *E. coli* biotin carboxylase exhibits an extreme form of negative cooperativity called half-sites reactivity,^{15, 16} it is tempting to speculate that the conduit for communication between the subunits occurs along this 16-stranded antiparallel β -sheet.

E. coli biotin carboxylase displays a slow ATPase activity in the absence of biotin or biotinylated-BCCP. As a consequence, the structure of the wild-type enzyme in complex with MgATP alone could not be determined. However, the structure of an inactive mutant (E288K) protein was co-crystallized with ATP.¹⁷ In this structure, the B-domain rotated by $\sim 45^\circ$ relative to the A- and C- domains allowing it to close over the active site cleft [Fig. 3(a)]. A close-up view of the ATP binding pocket is provided in Figure 3(b). Key side chains involved in anchoring the ligand to the protein include Lys 116, Lys 159, Glu 201, His 236, and Lys 288. Further details concerning the active site pocket are presented below. This “closed” conformation with bound nucleotide has been subsequently observed in other biotin carboxylases^{18, 19} as well as in the biotin carboxylase domains of the pyruvate carboxylases from *Staphylococcus aureus* and *Rhizobium etli* cfn42.²⁰⁻²²

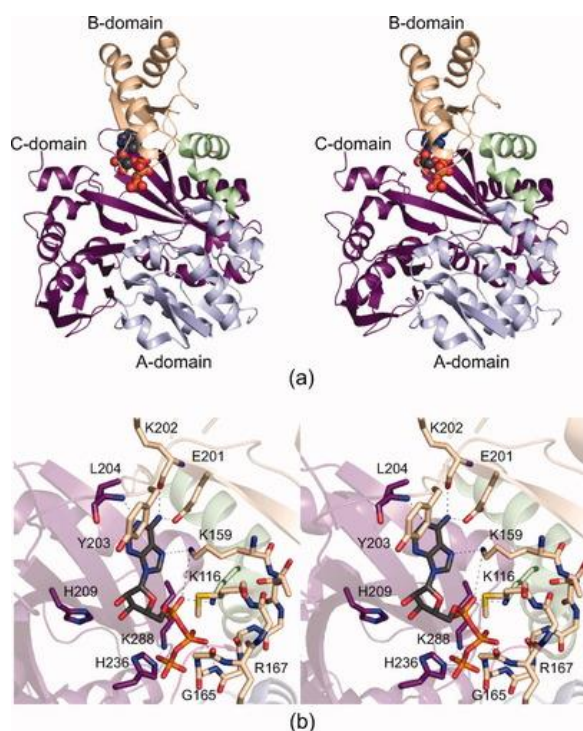


Figure 3 Structure of the *E. coli* biotin carboxylase in complex with ATP. A stereo-view of one subunit of the enzyme is presented in (a). The color-coding is the same as in Figure 2. The bound ATP is depicted in sphere representation. Note how the B-domain closes down upon nucleotide binding. Those amino acid residues important for ATP binding are shown in (b). The structure shown is that of the E288K mutant protein. Potential hydrogen bonds are indicated by the dashed lines. X-ray coordinates were obtained from the Protein Data Bank (1DV2).

One question that arises is whether the B-domains of the biotin carboxylase dimer are completely splayed open in the absence of nucleotides. In other words, do these domains only close down upon nucleotide binding? In the first structure of the *E. coli* biotin carboxylase, the B-domains of the dimer were completely open, albeit several residues in these domains were involved in crystal contacts. The crystal structure of the biotin carboxylase domain of the *Aquifex aeolicus* pyruvate carboxylase, however, also showed the B-domain in an open conformation.²³ In contrast, crystal structures of the apo biotin carboxylase domains from yeast acetyl-CoA carboxylase,²⁴ human acetyl-CoA carboxylase 2,²⁵ and *Bacillus thermodenitrificans* pyruvate carboxylase²⁶ all demonstrated conformations for the B-domains somewhere between the closed and completely open forms. Recent molecular dynamics studies of *E. coli* biotin carboxylase suggest that the most stable position for the B-domain may not be in the completely open state in the absence of substrate but rather in a partially closed state.²⁷ Single molecule fluorescence studies will be needed to experimentally confirm the resting positions of the B-domains in the absence of substrates.

The solution of the crystal structure of *E. coli* biotin carboxylase¹⁴ in the mid-1990s turned out to be fortuitous in that it became one of the charter members (along with glutathione synthetase,²⁸ D-Ala-D-Ala ligase,²⁹ and succinyl-CoA synthetase³⁰) in the discovery of the ATP-grasp superfamily of enzymes.^{31, 32} Most, but certainly not all members of this family, catalyze bond formation between a carboxylate and an amino (or thiol) group where the first step involves the hydrolysis of ATP to activate a carboxylate-containing substrate and the second step involves a nucleophilic attack on the carbonyl carbon of the acylphosphate intermediate (Fig. 4). The manner in which the acylphosphate intermediate is stabilized in the active site of an ATP-grasp protein is still not well understood, however.³³

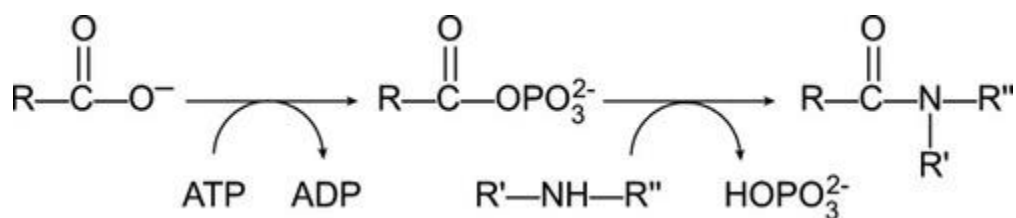


Figure 4 General overall reaction of enzymes belonging to the ATP-grasp superfamily. Most members employ an acylphosphate intermediate in their reaction mechanisms.

From structural studies, it is now known that all enzymes belonging to the ATP-grasp superfamily adopt similar molecular architectures with three major motifs: the N-terminal, the central, and the C-terminal regions. These motifs are also referred to in the literature as the A-, B-, and C-domains as described previously for biotin carboxylase [Fig. 2(a)]. Typically, the B-domain in an ATP-grasp protein contains four strands of antiparallel β -sheet flanked on one side by two α -helices. There is a highly flexible loop, typically glycine and serine rich, which connects the second and third β -strands of the B-domain [Fig. 5(a)]. This so-called T-loop, disordered in the absence of a nucleotide, becomes ordered upon nucleotide binding where it provides hydrogen-bonding interactions to the β - and γ -phosphoryl oxygens of the cofactor [Fig. 5(b)].³⁴ The orientation of the B-domain, with respect to the rest of the protein, depends upon the presence or absence of bound nucleotide in the binding cleft. When the nucleotide is present, the B-domain moves toward the A- and C-terminal regions due to the interactions of the nucleotide with residues of the T-loop: hence the name ATP-grasp. The A- and C-domains cluster together to provide the substrate-binding pocket [Fig. 5(c)]. Note that ATP-grasp proteins do not contain the canonical “P-loops” found in such enzymes as adenylate kinase, the G-proteins, and the myosin motor domain.³⁵ P-loops require the consensus sequence of Gly-X-X-X-Gly-Lys-Thr/Ser, and their structures are essentially insensitive to the presence or absence of ligands. This is in sharp contrast to the T-loops in the ATP-grasp proteins, in which their conformations are dependent upon the presence or absence of nucleotides and the nature of the moiety occupying the γ -phosphate position.³⁶ As such, the terminology of “P-loop” should not be applied to ATP-grasp proteins.

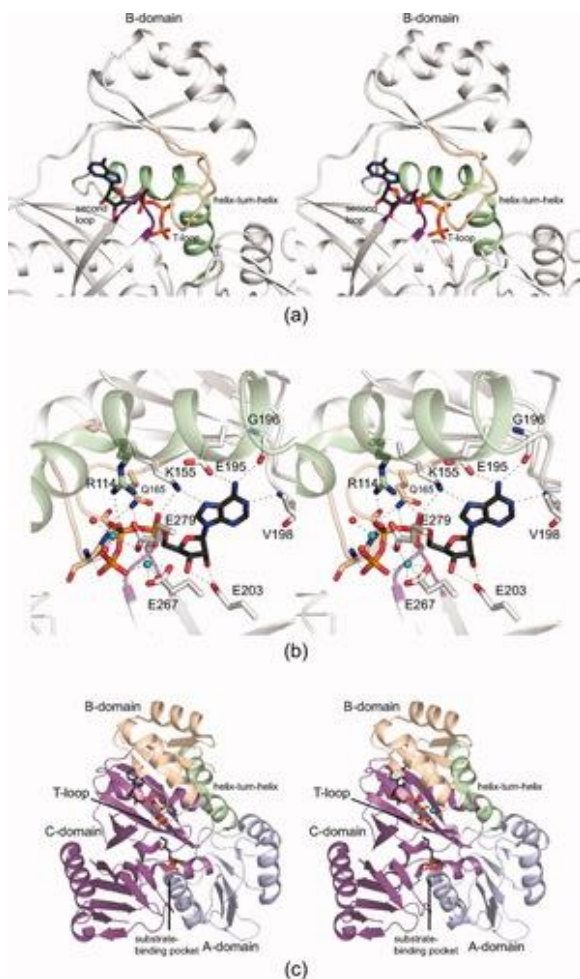


Figure 5 Elements of the ATP-grasp fold. The three-dimensional structure of PurT-encoded glycnamide ribonucleotide transformylase serves as a prototype for ATP-grasp superfamily members.^{36, 95} This enzyme, from *E. coli*, is involved in the *de novo* pathway for purine biosynthesis. All ATP-grasp superfamily members have B-domains that change their positions depending upon the presence or absence of bound nucleotides. In addition, as indicated in (a), they all have a characteristic helix-turn-helix motif that connects the A- and B-domains (light green) and a T-loop that surrounds the phosphoryl oxygens of the nucleotide triphosphate (wheat). A close-up view of the ATP-binding pocket for glycnamide ribonucleotide transformylase is presented in (b). One of the residues that interacts with the phosphoryl oxygens, Arg 114, is provided by the helix-turn-helix motif. ATP-grasp enzymes typically coordinate two magnesium ions with a glutamate residue serving as the metal bridging ligand. In the case of glycnamide ribonucleotide transformylase, this bridging ligand is Glu 279. The magnesium ions are depicted as teal spheres. Whereas the B-domain is intimately involved in nucleotide binding, the A- and C-domains form a cradle that harbors the side chains responsible for substrate binding, which for glycnamide ribonucleotide transformylase is glycnamide ribonucleotide (c).

Biotin Carboxylase Domains Bound to Substrates: Insights into Mechanism

Following the initial X-ray investigations of the *E. coli* biotin carboxylase, additional structures were subsequently determined in the presence of various ligands. Taken together these models have provided a detailed molecular scaffold for understanding the intriguing catalytic mechanism of the enzyme. We will begin our discussion of these complexes by first focusing on biotin carboxylase with its bound substrates, ATP, bicarbonate, and biotin, and then turning our attention to the binding modes for its inhibitors.

A variety of biotin carboxylase structures with bound nucleotides or nucleotide analogs have now been reported.¹⁷⁻¹⁹ From these studies, several key interactions between the protein and the nucleotides have been identified, many of which have been confirmed by site-directed mutagenesis. Figure 3(b) summarizes the most important of these interactions. Unless otherwise noted, the following discussion refers to the *E. coli* enzyme. The adenine moiety of ATP interacts with a loop of residues delineated by Glu 201 to Leu 204. The exocyclic amine forms hydrogen bonds with the carboxylate group of Glu 201 and the main chain carbonyl oxygen of Lys 202, whereas the peptidic nitrogen of Leu 204 interacts with N-1' of the adenine ring. In addition, Tyr 203 participates in a T-shaped stacking interaction with the adenine ring. Both Tyr 203 and Leu 204 form part of the hinge that allows the B-domain to rotate over the active site.^{17, 37} In some of the reported structures, the C-2 hydroxyl group of the ATP ribose lies within hydrogen bonding distance to the side chain of His 209. The importance of this interaction is underscored by the fact that the H209A mutant protein has a K_m for MgATP that is 70-fold higher than observed for the wild-type enzyme.³⁸ It should be noted that the homologous residue to His 209 in other members of the ATP-grasp superfamily is a glutamate. Another important active site interaction occurs between Lys 116 and the oxygens of the α - and β -phosphoryl groups of the nucleotide. Lys 116 lies in the elbow region of the helix-turn-helix motif, which according to molecular dynamics studies, moves towards the active site upon MgATP binding.²⁷ Mutation of Lys 116 to a glutamine causes a 50-fold increase in the K_m for MgATP, which further underscores the importance of this residue in ligand binding.³⁸

One of the most important architectural features of the B-domain is the T-loop defined by Lys 159 to Met 169 that covers the phosphate groups upon MgATP binding [Fig. 3(b)]. In models of biotin carboxylase with bound nucleotide, Lys 159 typically interacts with both N-7 of the purine base and with one of the α -phosphoryl oxygens of the nucleotide. Consistent with the X-ray crystallographic data, mutagenesis of Lys 159 to glutamine results in almost a 100-fold increase in the K_m for MgATP.³⁸ The T-loop contains a conserved sequence of amino acids (Ala-Ser-Gly-Gly-Gly-Gly-Arg-Gly), with the peptidic groups of two of the glycines, Gly 165 and Gly 166, interacting with a β -phosphoryl oxygen of the ligand [Fig. 3(b)]. Site-directed mutagenesis of these two residues to valines yielded enzymes with 40-fold increases in the K_m for MgATP.³⁹

The biotin carboxylase domains of biotin-dependent proteins are commonly referred to as “two metal enzymes” because two metal ions are required for their catalytic activities. The metals used by biotin carboxylases *in vivo* are magnesium ions, but at least for the *E. coli* biotin carboxylase, manganese and cobalt can also support activity.⁴⁰ A close-up view of the coordination spheres surrounding the magnesium ions when bound to the *S. aureus* biotin carboxylase is provided in Figure 6(a). This structure was determined in the presence of the non-hydrolyzable ATP analog AMPPNP.¹⁸ As mentioned previously, one of the metal ions binds to the enzyme as the metal-nucleotide chelate. It is not possible to determine which magnesium ion in Figure 6(a) represents the “metal” in the metal-nucleotide chelate. However, it should be noted that magnesium is mostly coordinated to the β - γ phosphoryl groups of ATP suggesting that magnesium (A) is part of the ATP substrate.⁴¹ Both magnesium ions are octahedrally ligated and bridged by the side chain of Glu 288. Magnesium “A” is surrounded by β - and γ -phosphoryl oxygens of ATP, the carboxylate side chain of Glu 288 (a bidentate ligand), and two water molecules. In contrast, magnesium (B) is coordinated by the side chain carboxylate of Glu 274 (a bidentate ligand), oxygen atoms from the α - and γ -phosphoryl groups, a water molecule, and a carboxylate oxygen contributed by Glu 288. The coordination geometry exhibited by the *S. aureus* biotin carboxylase has also been observed in the biotin carboxylase domain of pyruvate carboxylase¹⁶ and in other enzymes belonging to the ATP-grasp superfamily including carbamoyl phosphate synthetase,⁴² glycylamide ribonucleotide transformylase,³⁶ and *N*⁵-carboxyaminoimidazole ribonucleotide synthetase,^{43, 44} among others.

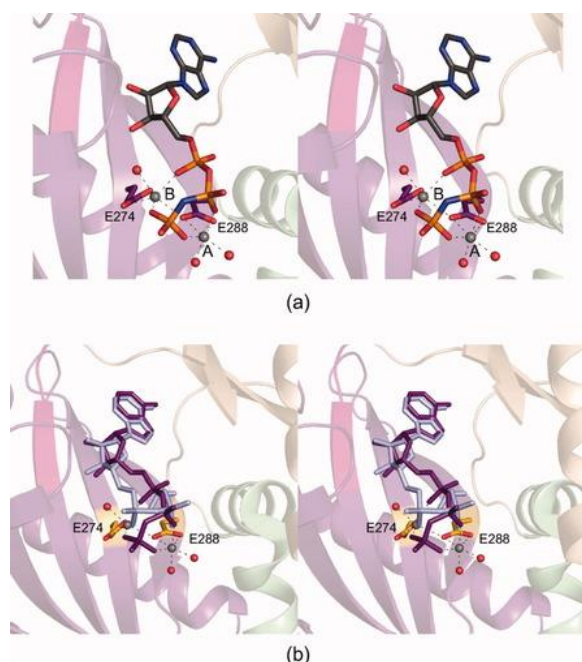


Figure 6 The binding of MgAMPPNP to the *S. aureus* biotin carboxylase. Shown in (a) is a close-up view of the region surrounding the MgAMPPNP ligand when bound to the enzyme (PDB accession no. **2VPQ**). Both magnesium ions, depicted as gray spheres, display octahedral coordination geometry. Water molecules are shown as red spheres. The differences in AMPPNP binding, with or without magnesium ions, is shown in (b). The nucleotide, with its accompanying metal ions, is displayed in violet. Superimposed on this is the conformation of the nucleotide when it binds into the active site without its accompanying metals (light blue, PDB accession no. **2J9G**).

The role of the metal ions in catalysis is, most likely, to correctly position MgATP into the active site cleft. In the structure of the *E. coli* biotin carboxylase complexed with AMPPNP in the absence of metals, the phosphate chain curls back onto itself.¹⁸ This unusual conformation of AMPPNP is in stark contrast to the extended phosphate chain observed when both magnesium ions are present [Fig. 6(b)]. In addition to the X-ray structural data, site-directed mutagenesis of the two active site residues coordinating the metals, Glu 274 and Glu 288, results in a 100-fold decrease in maximal velocity, thus suggesting that any alteration in the positioning of ATP dramatically affects catalysis.^{38, 45} In addition to the structural role of the metal ions, there is also a well-established kinetic function. Attwood and others have shown that in the biotin carboxylase domain of pyruvate carboxylase, Mg^{2+} stabilizes the enzyme-carboxybiotin complex.^{8, 46}

Thus far, two crystal structures of biotin carboxylase with biotin and ADP have been reported. Chou *et al.*,¹⁹ described the structure of *E. coli* biotin carboxylase with bound bicarbonate, free biotin, and ADP, whereas Lietzan *et al.*,²² determined the structure of *R. etli* pyruvate carboxylase with BCCP-biotin and ADP bound in the biotin carboxylase domain. In the structure of *E. coli* biotin carboxylase, bicarbonate is anchored into the active site through interactions with Arg 292, Glu 296, and Arg 338 [Fig. 7(a)]. On the basis of this model with the fully loaded active site, it was postulated that Glu 296 functions as an active site base to remove the proton from bicarbonate. The E296A mutant protein demonstrated only a 10-fold decrease in the activity; however,¹⁹ it was argued that in the mutant protein a hydroxide ion removes the proton. It is hard to reconcile how the enzyme generates such a hydroxide ion given the high pK of water. Furthermore, the R292A mutant protein showed no increase in the K_m for bicarbonate, thus calling into question whether the observed bicarbonate-binding site in the crystalline protein is an artifact.⁴⁵ It is also important to note that in the original structure of *E. coli* biotin carboxylase, a phosphate, derived from the crystallization buffer, was found in the active site. The phosphate group also participates in electrostatic interactions with Arg 292, Glu 296, and Arg 338. An overlay of the two

biotin carboxylase complexes demonstrates that the bicarbonate and phosphate ligands lie virtually on top of one another [Fig. 7(b)]. Interestingly, the structure of the E296A mutant protein reported by Chou *et al.*,¹⁹ showed a sulfate ion, again derived from the crystallization solution, located in the putative bicarbonate-binding site.¹⁹ Perhaps the bicarbonate and the phosphate product exchange positions during the course of catalysis as proposed by Zeczycki *et al.*⁷ An alternative explanation, however, is that the observed binding position for the bicarbonate in the enzyme/biotin/bicarbonate/ADP crystalline complex does not represent the “true” bicarbonate binding pocket in a productive enzyme complex because ATP and its associated metals are missing.

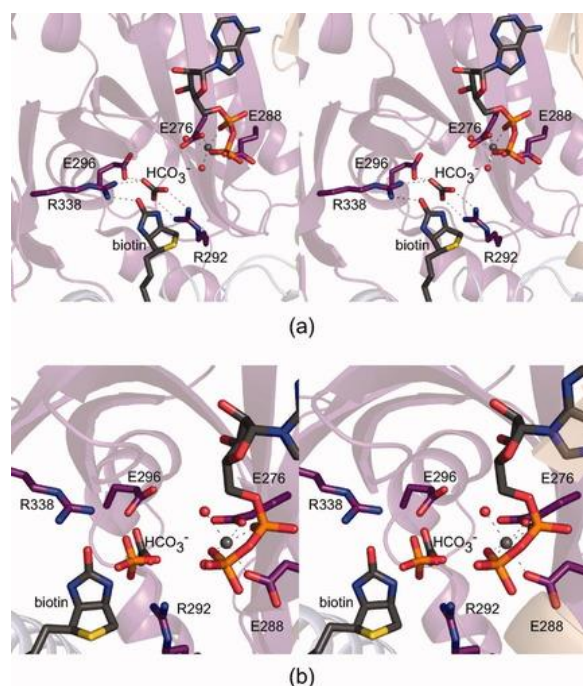


Figure 7 The binding of biotin, bicarbonate, and MgADP to the *E. coli* biotin carboxylase. A stereo-view of the biotin carboxylase active site with its bound ligands is shown in (a). Potential hydrogen bonds are indicated by the dashed lines. An overlay of the structure of biotin carboxylase solved in the presence of biotin, bicarbonate, and MgADP with that determined only in the presence of inorganic phosphate is shown in (b). The bicarbonate and phosphate are located in nearly identical positions in the active sites of the two models. X-ray coordinates were obtained from the Protein Data Bank (**3G8C** and **1BNC**).

There are additional discrepancies between the X-ray crystal structure of the fully loaded structure of the *E. coli* biotin carboxylase and subsequent solution studies. As can be seen in Figure 7(a), Arg 338 lies within hydrogen bonding distance to the carbonyl oxygen of the cofactor,¹⁹ and it was suggested that it stabilizes the oxyanion formed during the carboxylation reaction. Two different site-directed mutagenesis studies targeting Arg 338 (R338Q, R338S, and R338A)^{25, 47} found that the K_m for biotin does not change, but the maximal velocity decreases anywhere from 10-fold to 230-fold. One investigation reported the K_m for ATP increased 63-fold in the Arg 338 mutant proteins.⁴⁷ This same study also demonstrated that these Arg 338 mutant proteins exhibited negative cooperativity with respect to bicarbonate. Mutations of the homologous arginine in *R. etli* pyruvate carboxylase resulted in a 25- to 100-fold decrease in the maximal velocity. Analysis of the biotin carboxylase domain of *R. etli* pyruvate carboxylase supports a role for this arginine in stabilizing the biotin enolate in the enzyme active site and suggests that an additional role may be to prevent the return of carboxybiotin to the biotin carboxylase domain active site.^{7, 22} These results are especially intriguing considering that the original biotin carboxylase structure showed that Arg 338 interacts with phosphate, which binds in the putative bicarbonate-binding site. A crystal structure of biotin carboxylase in complex with ATP, bicarbonate, biotin and

two magnesium ions is critically needed in order to reconcile the observed differences between solid state and solution studies.

The structural analyses of Chou *et al.*,¹⁹ represented significant advances in our understanding of the reaction mechanism of biotin carboxylase. Nonetheless, in light of the fact that phosphate and sulfate ions can occupy the “bicarbonate-binding” site, that the kinetic data on the R292A and E296A mutant proteins do not strongly support the proposed mechanism, and that the structure reported by Chou *et al.*,¹⁹ contained ADP, not ATP, the jury is still out regarding the catalytic mechanism of biotin carboxylase. It is possible that the mechanism of biotin carboxylase proceeds in a manner more similar to that proposed for *N*⁵-carboxyaminoimidazole ribonucleotide synthetase.⁴⁴ For this enzyme it is thought that an active site base is not required to deprotonate bicarbonate. Rather bicarbonate, in its protonated form, reacts with ATP to generate a carboxyphosphate intermediate, which adopts a pseudochain conformation as suggested by molecular mechanics calculations.⁴³ According to the proposed mechanism, the carboxyphosphate decomposes to CO₂ via an intramolecular proton abstraction by the phosphate group.⁴⁴

Recently, the Tong group reported a structure of *E. coli* biotin carboxylase with two ADP molecules bound in the active site.⁴⁸ One nucleotide was in the ATP-binding site whereas the other one was located in the bicarbonate and biotin-binding site. This model was used to explain substrate inhibition by ATP in biotin carboxylase, and the authors proposed that inhibition by ATP was how the enzyme was regulated.

Biotin Carboxylase Domains Bound to Inhibitors

The very recent discovery of inhibitors to prokaryotic biotin carboxylases represents a classical example of serendipity. Investigators at Pfizer were screening bacteria against a library composed of inhibitors to tyrosine protein kinases and found that the pyridopyrimidines [Fig. 8(a)] possess antibacterial activities.⁴⁹ To identify the enzymological target of these compounds, resistant strains of *Hemophilus influenza* were generated, and the resistance mapped to the gene coding for biotin carboxylase. X-ray crystallographic and enzyme inhibition analyses of the pyridopyrimidines showed that they bind in the ATP site and interact with many of the same residues as the nucleotide, namely the loop of residues between Glu 201 to Leu 204 and Lys 159 [Fig. 8(b)].⁵⁰

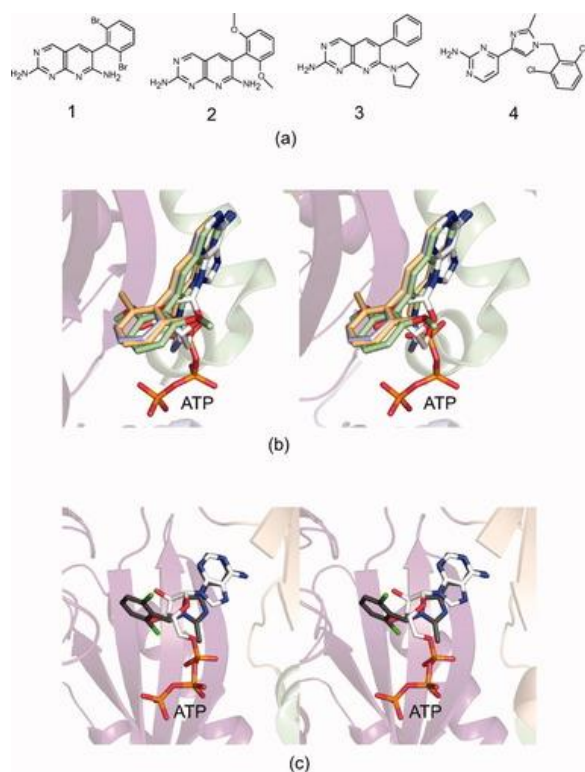


Figure 8 The binding of pyridopyrimidines and aminooxazoles to biotin carboxylase. The chemical structures for three pyridopyrimidine inhibitors of biotin carboxylase (1, 2, and 3) and one aminooxazole (4) are shown in (a). The manners in which the pyridopyrimidine inhibitors bind to biotin carboxylase are depicted in stereo in (b). Compounds 1, 2, and 3 are highlighted in light orange, light green, and light blue, respectively. For comparison purposes, the conformation of ATP, when bound to biotin carboxylase, is also shown. Compound 4 binds in a similar orientation to the pyridopyrimidine inhibitors as depicted in (c). X-ray coordinates were obtained from the Protein Data Bank (**2V58**, **2V59**, **2V5A**, **2W71**, and **1DV2**).

After validating biotin carboxylase as a target for antibacterial agents, the Pfizer group capitalized on the biotin carboxylase/pyridopyrimidine structures by conducting virtual screening and fragment-based drug design to develop additional classes of inhibitors.⁵¹ Figure 8(c) shows an example of one of the biotin carboxylase inhibitors discovered by this approach. It belongs to the aminooxazole series of compounds, which have pharmacophore features very similar to the pyridopyrimidines. As expected, these types of inhibitors bind to the ATP site and utilize similar interactions to anchor to the protein.

At the same time the Pfizer group was working with the pyridopyrimidines and aminooxazole inhibitors, scientists at Schering-Plough utilized high-throughput screening to discover that benzimidazole-carboxamide derivatives also inhibit bacterial biotin carboxylases [Fig. 9(a)].⁵² These types of compounds bind in the ATP pocket using the same amino acid residues as the pyridopyrimidines and aminooxazoles [Fig. 9(b)].

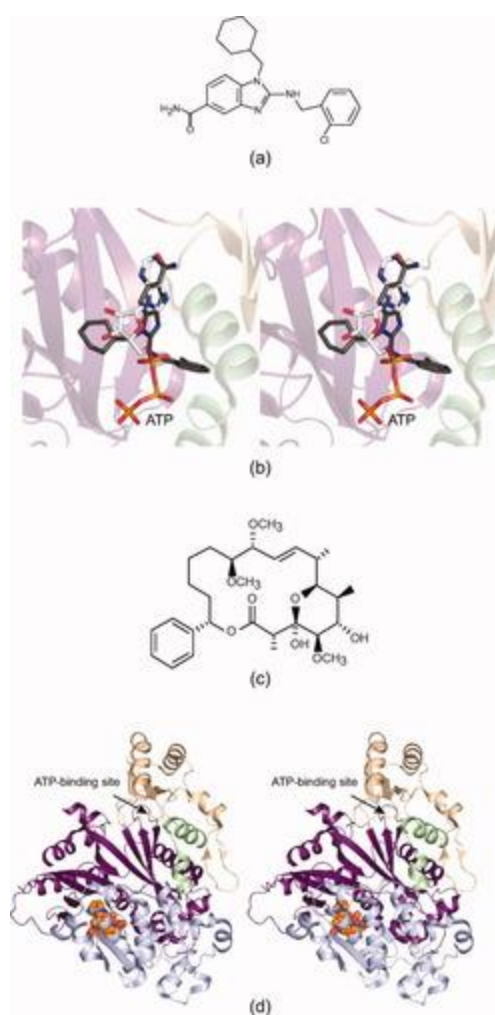


Figure 9 The binding of inhibitors to biotin carboxylase. The chemical structure of 2-(2-chlorobenzylamino)-1-(cyclo-hexylmethyl)-1H-benzo[d]imidazole-5-carboxamide is shown in (a). The manner in which this compound binds to biotin carboxylase is depicted in (b). On the basis of this initial structural analysis, a series of novel inhibitors was created.⁵² X-ray coordinates used were from the Protein Data Bank (**3JZF**). Soraphen A is a potent antifungal agent with the chemical structure shown in (c). It binds to yeast biotin carboxylase in a pocket located at the interface between the N- and C-terminal domains as indicated by the space-filling representation in (d). X-ray coordinates were from the Protein Data Bank (**1W96**).

Unlike the prokaryotic biotin carboxylases, there is only one inhibitor of eukaryotic biotin carboxylases that has been identified thus far, which is soraphen A [Fig. 9(c)]. It is a natural product that displays antifungal activity.⁵³ The structure of yeast biotin carboxylase bound to soraphen A shows that the inhibitor is 25 Å away from the active site and positioned at the interface between the A- and C-terminal domains [Fig. 9(d)].²⁴ Crystals of the yeast enzyme, in complex with soraphen A, contain three subunits in the asymmetric unit, and these subunits demonstrate neither dimeric nor trimeric associations. It is thought that soraphen A functions by inhibiting dimerization.

Recent structural analyses of the biotin carboxylase domain of human mitochondrial acetyl-CoA carboxylase have also shown that soraphen A binds in the same position as that observed for the yeast enzyme.⁵⁴ The soraphen A binding site in the human enzyme is also the phosphorylation site used for regulation of its activity.^{54, 55} Phosphorylation of the enzyme prevents polymerization of the inactive monomers into the active long filamentous form. The fact that soraphen A binds in the same site in which phosphorylation occurs suggests that it inhibits enzyme activity by preventing polymerization.⁵⁴

Structure of the Biotin Carboxyl Carrier Protein

The ability of the biotin carboxylase and carboxyltransferase components of *E. coli* acetyl-CoA carboxylase to use free biotin as a substrate greatly simplified the analysis of these two enzymes. The fact remains, however, that biotin is not the natural substrate. *In vivo*, biotin is covalently attached to the biotin carboxyl carrier protein (BCCP). In some bacteria (e.g. *E. coli*) BCCP is a separate protein, whereas in other prokaryotes biotin carboxylase and BCCP are on a single polypeptide chain. The situation in eukaryotes is completely different; BCCP is on the same polypeptide chain as the biotin carboxylase and carboxyltransferase functionalities.

Early structural analyses on BCCP focused on the protein from *E. coli*, which is composed of two domains, the N-terminal and C-terminal. The C-terminal domain contains biotin covalently attached to a specific lysine (Lys 122). Before the gene for *E. coli* BCCP was cloned, conventional purification of BCCP sometimes resulted in proteolytic cleavage between these two domains. Athappilly and Hendrickson took advantage of this property by purifying BCCP, cleaving it with subtilisin Carlsberg to generate the biotinylated C-terminal domain (hereafter referred to as BCCPsc), and crystallizing it.⁵⁶ The structure of this C-terminal domain was solved to a nominal resolution of 1.8 Å.

As can be seen in Figure 10, the structure of the biotinylated C-terminal domain consists of two four-stranded antiparallel β -sheets arranged around an approximate two-fold rotational axis. The α -carbons for the two halves superimpose with a root-mean-square deviation of 0.52 Å.⁵⁶ The lysine residue to which biotin is attached is located in a loop connecting two of the β -strands. The C-terminal domain of *E. coli* BCCP also contains a “thumb-like” structural motif lying near the biotin moiety. This thumb contains Thr 94 with its side and main chain oxygen atoms lying within ~ 3 Å of the carbonyl oxygen and N1' of biotin, respectively. On the basis of these distances, it was proposed that Thr 94 forms hydrogen-bonding interactions with biotin.

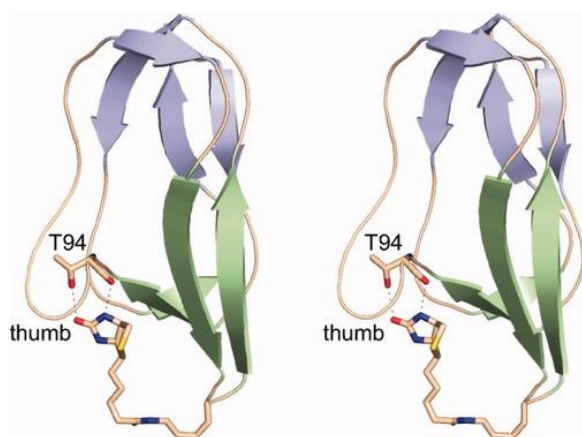


Figure 10 The molecular architecture of the C-terminal domain of the *E. coli* BCCP. The protein is characterized by two layers of antiparallel β -sheet, highlighted in blue and green. Presumed hydrogen bonds between Thr 94 and biotin are indicated by the dashed lines. Subsequent NMR studies suggest that these hydrogen bonds do not occur. X-ray coordinates were from the Protein Data Bank (**1BDO**).

At the same time the crystal structure of the biotinylated C-terminal domain of *E. coli* BCCP was published, John Wallace and coworkers reported the cloning, overexpression, and purification of the BCCP C-domain containing 87 rather than 80 residues (hereafter referred to as BCCP87).⁵⁷ BCCP87 was found to be an excellent substrate for both biotin carboxylase and carboxyltransferase,⁵⁸ and its small size made it amenable to a structural analysis by NMR.⁵⁹ Not surprisingly, the NMR structures of both the apo and holo forms of BCCP87 were very similar to the crystal structure of BCCPsc with a few notable exceptions.⁶⁰ Most importantly, solvent deuterium exchange experiments revealed that Thr 94 does not, in fact, form hydrogen bonds with the N1' of biotin as originally

proposed.⁵⁶ Several NOEs (i.e., interactions) were detected, however, between the biotin moiety and the amino acid residues in the “thumb-like” region. Most likely, these residues serve to immobilize the biotin cofactor.

In contrast to the three-dimensional structure of the biotinoyl domain of *E. coli* BCCP, the structures of the biotin containing 1.3S subunit of transcarboxylase⁶¹ and the biotinoyl domain of human acetyl-CoA carboxylase⁶² do not contain the “thumb-like” motif. Yet, their overall folds are very similar to that of *E. coli* BCCP. Because both proteins are small, NMR was used to determine their structures, thus allowing their dynamical properties to be analyzed. Unlike *E. coli* BCCP87, no NOEs between the biotin moiety and the protein were detected in either protein. Given that both the 1.3S subunit of transcarboxylase and the biotinoyl domain of human acetyl-CoA carboxylase appear to function quite well without a “thumb,” the question arises as to the role of this motif in the *E. coli* BCCP. Mutations in this region of the *E. coli* BCCP were found to impair the growth of the bacterium suggesting that it is important for the function of the protein, but the chemical mechanism for this phenotype is not understood.⁶³ In order to address the role of this motif, Weaver *et al.*,⁶⁴ modeled holo BCCP87 onto the structure of biotin carboxylase with ATP bound and proposed that the major point of contact was between a β -strand in BCCP87 (Gln 122 to Gln 126) and a β -strand in the B-domain of biotin carboxylase (Gly 168 to Val 172). Whether this modeling is correct or not will have to await additional structural studies of BCCP bound to biotin carboxylase and carboxyltransferase.

Carboxyltransferase Domains with the Crotonase Fold

Any discussion of the carboxyltransferase domain structure must necessarily be given in context of the biochemical problem that the enzyme has to solve. The major chemical hurdle in the second half-reaction of biotin-dependent enzymes is the stabilization of enolate anions. For instance, in the Class I carboxylases, CO₂ is transferred from carboxybiotin to an acceptor molecule. As shown in Scheme 4, this transfer requires an enolate anion in the acceptor molecule as well as an enolate-like anion in biotin. Since enolate anions abound in biochemical systems, one particular strategy that nature has evolved utilizes two hydrogen bonds from peptidic NH groups to stabilize oxyanion moieties. These peptidic NH groups form an “oxyanion” hole. One such protein scaffold that correctly juxtaposes peptidic NH groups to form an oxyanion hole is referred to as a crotonase (or enoyl-CoA hydratase) fold after the enzyme for which it was first described.^{65, 66} Members of the crotonase superfamily contain a core domain of repeated $\beta\beta\alpha$ motifs that form two parallel β -sheets situated at 90° with respect to each other. These β -sheets are surrounded by α -helices. A ribbon representation of enoyl-CoA hydratase, the founding member of the superfamily, is displayed in Figure 11(a). One of the two residues providing the peptidic NH groups for the oxyanion hole is positioned at the N-terminus of an α -helix, and it is thought that the positive charge of the helix dipole also contributes to transition-state stabilization [Fig. 11(b)].

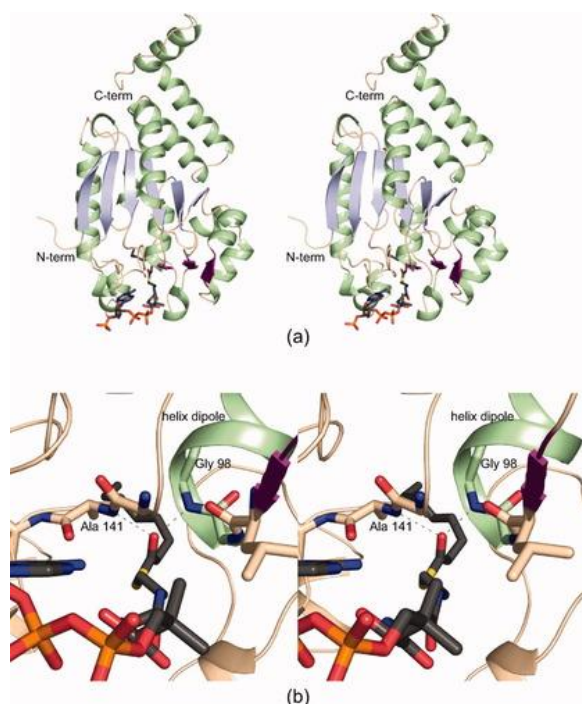
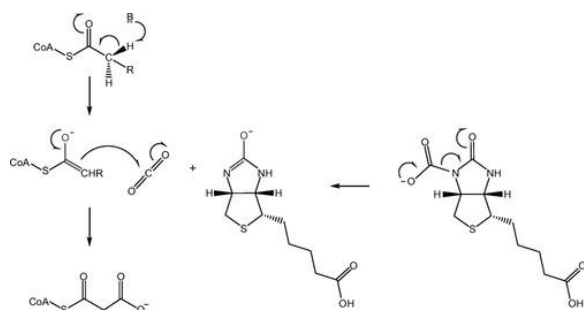


Figure 11 The structure of enoyl CoA hydratase. Members of the crotonase superfamily typically display either trimeric or hexameric quaternary structures with their active sites wedged between subunits. Shown in (a) is a ribbon representation of one subunit of enoyl CoA hydratase, an enzyme that catalyzes the second step in fatty acid oxidation. X-ray coordinates were from the Protein Data Bank (**2DUB**).⁷⁷ The subunit can be envisioned in terms of two domains. The N-terminal region is dominated by two layers of β -sheet (displayed in blue and magenta), whereas the C-terminal motif contains three α -helical regions (all α -helices are shown in green). Members of the crotonase superfamily contain an oxanion hole, which is formed by two peptidic nitrogen groups (b). Also, in these family members, it is thought that a helix dipole moment plays a role in activating the carbonyl carbons of the substrates for nucleophilic attacks.



Scheme 4

The carboxyltransferase domains of biotin-dependent enzymes must stabilize enolate anions in both substrates. It is thus not surprising that their three-dimensional structures are composed of homologous domains (or polypeptide chains depending upon the protein being discussed). These domains have the crotonase fold, and they form the basic architectural element for the various quaternary structures described below. Indeed, from the following descriptions, it is clear that nature started with the crotonase fold, and then evolved it into ever more complex quaternary structural arrangements.

E. Coli and *S. Aureus* Carboxyltransferases

The carboxyltransferase domains of the *E. coli* and *S. aureus* acetyl-CoA carboxylases are $\alpha_2\beta_2$ heterotetramers [Fig. 12(a)].⁶⁷ Although the crystal structures were solved in the absence of bound substrates, site-directed mutagenesis and solution studies indicate that the α -subunits bind biotinylated-BCCP, whereas the β -subunits bind acetyl-CoA. The active sites lie at the interfaces between the α,β dimers [Fig. 12(a)]. In addition, both the α - and β -subunits have a crotonase-like fold, thus suggesting that the genes encoding them arose from a duplication event.

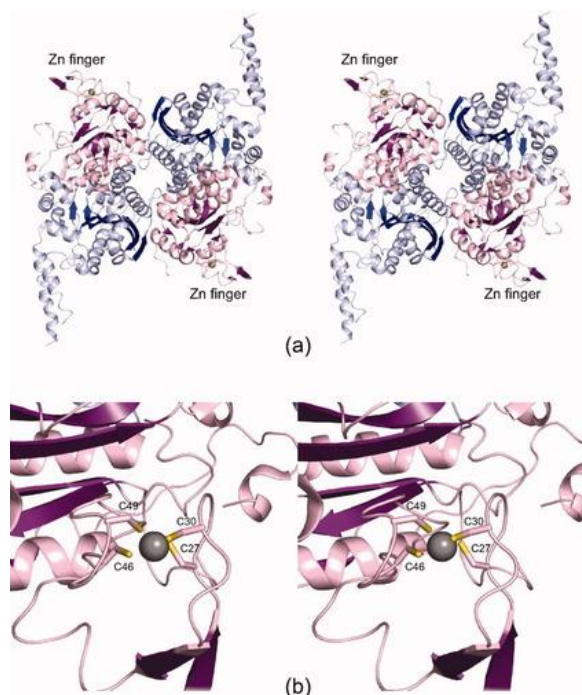


Figure 12 The structure of the *E. coli* carboxyltransferase. The quaternary structure of the enzyme is an $\alpha_2\beta_2$ heterodimer. X-ray coordinates were from the Protein Data Bank (2F9Y). The α - and β -chains are colored in light blue and pink, respectively in (a). The view shown is looking down the twofold rotational axis that relates one α,β dimer to another. Each β -subunit contains a four-cysteine zinc finger as shown in (b).

The crystal structures of the *E. coli* and *S. aureus* carboxyltransferases revealed an unexpected feature not observed in other carboxyltransferases, namely a four-cysteine zinc finger domain on the N-terminus of the β -subunit [Fig. 12(b)].⁶⁷ The zinc finger is part of a saddle-like motif that is characterized by a patch of amino acids with a positive electrostatic surface potential. The rest of the protein has an overall negative electrostatic surface potential.⁶⁷ Zinc finger domains are commonly associated with proteins that bind nucleic acids, and, indeed, the *E. coli* and *S. aureus* carboxyltransferases were subsequently shown to bind DNA and RNA.^{68, 69} Using electrophoretic mobility shift assays, it was demonstrated that the *E. coli* carboxyltransferase specifically binds to the mRNA molecules encoding the α - and β -subunits, and this binding results in the inhibition of translation. In addition, the acetyl-CoA substrate was shown to compete with the two mRNA molecules for binding to carboxyltransferase.⁷⁰ These findings led to an intriguing model for a negative feedback loop in the regulation of carboxyltransferase activity and ultimately for the regulation of fatty acid biosynthesis in *E. coli*.^{70, 71} Briefly, in stationary phase, when acetyl-CoA levels are low, carboxyltransferase acts as a “dimmer switch” by binding to the mRNA coding for the α and β subunits and inhibiting translation as well as enzymatic activity. In contrast, during log phase the high concentration of acetyl-CoA competes with mRNA for binding to carboxyltransferase, thereby allowing catalysis to occur as well as translation of mRNA to synthesize more carboxyltransferase.

Yeast Carboxyltransferase and the α -Subunit of Glutaconyl-CoA Decarboxylase

The carboxyltransferase component of acetyl-CoA carboxylase from yeast is a dimer with each monomer consisting of two domains [Fig. 13(a)]. The crystal structure showed that the N-terminal domain contained CoA and therefore is most likely to bind the substrate acetyl-CoA, whereas the C-terminal domain binds the biotin substrate.⁷² Thus, the eukaryotic version of the carboxyltransferase subunit appears to be a fusion of the α - and β -subunits of the prokaryotic carboxyltransferase. The two monomers of the yeast enzyme are arranged in a head-to-tail arrangement such that the active site is at the interface between the N-terminal domain of one monomer and the C-terminal domain of another monomer [Fig. 13(a)].

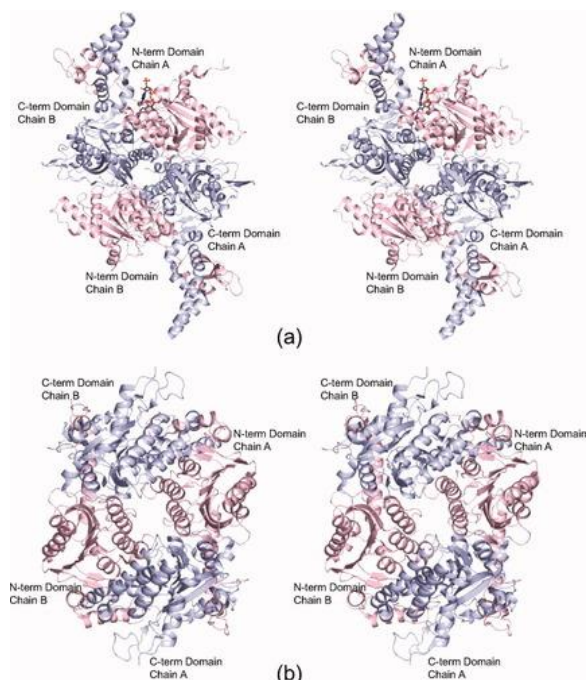


Figure 13 The carboxyltransferase domain of yeast acetyl-CoA carboxylase and the α -subunit of glutaconyl-CoA decarboxylase demonstrate similar quaternary structures. Both function as homodimers. Shown in (a) is the carboxyltransferase of the yeast acetyl-CoA carboxylase. X-ray coordinates were from the Protein Data Bank (**1OD2**). Each subunit of the homodimer can be envisioned in terms of an N-terminal and a C-terminal domain, displayed in pink and blue, respectively. Likewise, the two α -subunits of the glutaconyl-CoA decarboxylase dimer adopt similar bilobal type architectures. The N- and C-terminal domains are depicted in pink and blue, respectively. X-ray coordinates were from the Protein Data Bank (**1PIX**).

A similar type of quaternary structure has also been observed in the α -subunit of glutaconyl-CoA decarboxylase from *Acidaminococcus fermentans*.⁷³ This subunit catalyzes the first reaction in Scheme 2 where CO_2 is transferred from glutaconyl-CoA to biotin. As in the yeast carboxyltransferase, the α -subunit of glutaconyl-CoA decarboxylase functions as a homodimer with each monomer containing two crotonase-like domains. Each domain binds one of the two substrates. The two monomers are also arranged in a head-to-tail fashion with the active sites lying at the interfaces between the N-terminal and C-terminal domains of adjacent monomers [Fig. 13(b)].

Transcarboxylase 12S Subunit and the β -Subunit of Propionyl-CoA Carboxylase

The quaternary structures of the transcarboxylase 12S subunit from *P. shermanii*⁷⁴ and the β -subunit of propionyl-CoA carboxylase from *Streptomyces coelicolor*⁷⁵ take the homodimeric structures found in yeast carboxyltransferase and glutaconyl-CoA decarboxylase and expand them into hexameric structures, which can be thought of as either trimers of dimers or dimers of trimers [Fig. 14(a,b)]. Each monomer in these two enzymes has two domains where the N-terminal region binds methylmalonyl-CoA for the transcarboxylase 12S subunit or propionyl-CoA for propionyl-CoA carboxylase and the C-terminal motif binds biotin. Like all the other carboxyltransferases discussed so far, both domains have a crotonase type fold. Each dimeric pair is oriented in a head-to-tail manner so that the active sites reside at the interface between opposing N- and C-terminal domains contributed by different monomers. Other enzymes in the crotonase superfamily also form hexamers with the same 32 symmetry observed in the transcarboxylase 12S subunit and the β -subunit of propionyl-CoA carboxylase. These include methylmalonyl-CoA decarboxylase,⁷⁶ enoyl-CoA hydratase (crotonase),⁷⁷ and dienoyl-CoA isomerase.⁷⁸

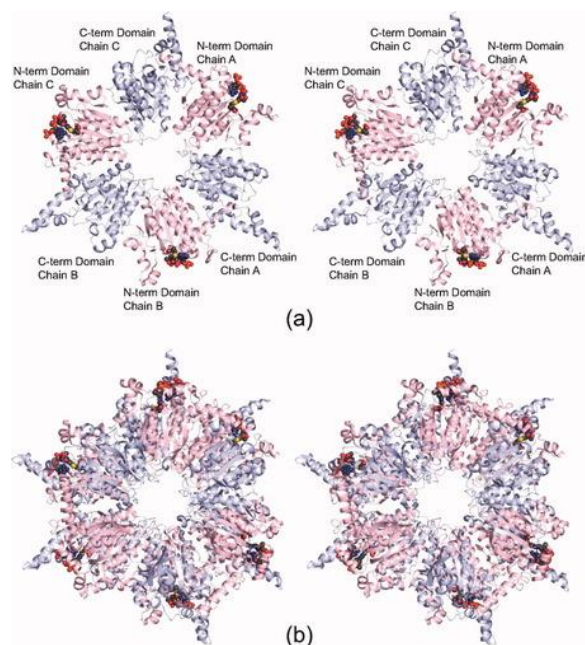


Figure 14 The transcarboxylase 12S subunit shows a hexameric quaternary structure. The quaternary structure of the enzyme can be envisioned as a dimer of trimers. X-ray coordinates were from the Protein Data Bank (1ON3). The trimer is shown in (a) with the methylmalonyl-CoA ligands displayed in sphere representations. Each monomer consists of an N- and a C-terminal domain, colored in pink and blue, respectively. The complete hexamer is shown in (b).

The transcarboxylase 12S subunit and the β -subunit of propionyl-CoA carboxylase are simply larger oligomeric assemblies of the basic didomain homodimeric structures observed for yeast carboxyltransferase and glutaconyl-CoA decarboxylase. One might ask why the latter two enzymes do not form such hexamers. It is a function of the extra four α -helices found at their C-termini. These α -helices, which are absent in the transcarboxylase 12S subunit and the β -subunit of propionyl-CoA carboxylase, prevent the formation of hexameric quaternary structures.

Active Site Architecture and Substrate Binding

Given the number of carboxyltransferase subunit or domain structures now solved, there is still a decided lack of three-dimensional information regarding the manner in which substrates bind to these enzymes. The structure of the yeast carboxyltransferase domain of acetyl-CoA carboxylase contained CoA, whereas the 12S subunit of transcarboxylase had bound methylmalonyl-CoA.^{72, 74} The best characterized, with respect to three-dimensional architecture, is the β -subunit of propionyl-CoA carboxylase from *S. coelicolor* in which the binding pockets for both propionyl CoA and biotin have been identified.⁷⁵

In all of these enzymes, the acyl-CoA/CoA ligands bind in bent or U-shaped conformations with their carbonyl oxygens residing in oxyanion holes. For the 12S subunit of transcarboxylase, the carbonyl oxygen of methylmalonyl-CoA, and by inference its enolate ion, forms hydrogen bonds to the peptidic NH group of Ala 143 and a water molecule [Fig. 15(a)].⁷⁴ The water molecule is held in place via hydrogen bonds to the peptidic NH group of Ala 183 and the main chain carbonyl oxygen of Ala 180. The oxyanion hole of the β -subunit of propionyl-CoA carboxylase is somewhat different. It is formed by the peptidic NH group of Gly 183 [Fig. 15(b)].⁷⁵ In addition, the carbonyl oxygen of propionyl-CoA lies within 3.2 Å of N-1' of biotin.

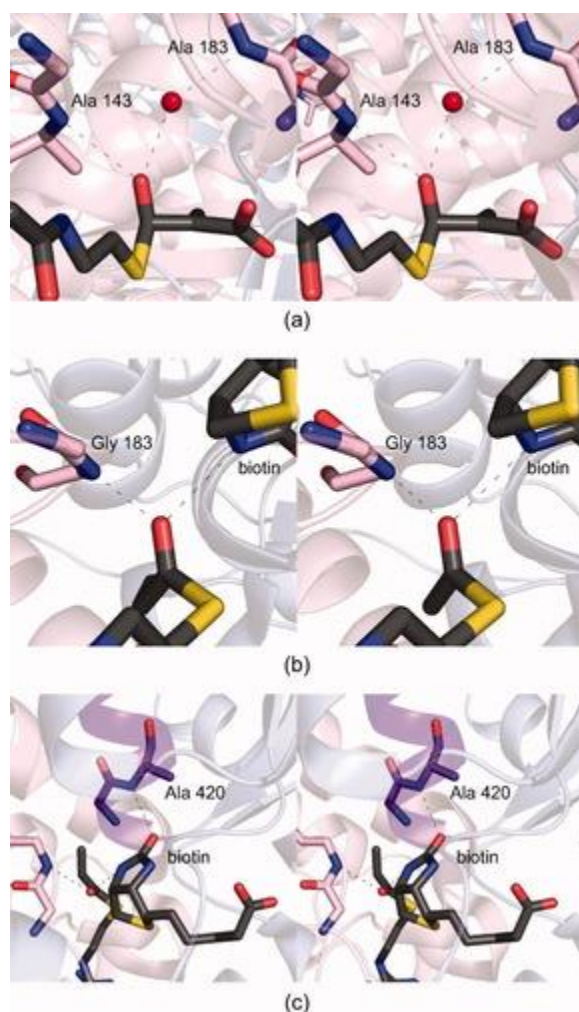


Figure 15 Oxyanion holes in carboxyltransferases. In the 12S subunit of transcarboxylase, the oxyanion hole is formed by the backbone amide group of Ala 143 and a water molecule as depicted in (a). X-ray coordinates were from the Protein Data Bank (**1ON3**). In contrast, in the β -subunit of propionyl-CoA carboxylase, one backbone amide group forms the oxyanion hole as shown in (b). X-ray coordinates were from the Protein Data Bank

(1XNY). The oxyanion hole in propionyl-CoA carboxylase is formed by the backbone amide group of Ala 420 and the positive end of a helix dipole moment. X-ray coordinates were from the Protein Data Bank (1XNY).

The structure of the β -subunit of propionyl-CoA carboxylase was especially informative because it contained biotin bound in the active site.⁷⁵ The biotin-binding site is lined with hydrophobic residues, which as pointed out by Diacovich *et al.*, is very similar to the hydrophobic and van der Waals interactions observed between biotin and streptavidin.⁷⁴ The carbonyl oxygen of the ureido ring binds in an oxyanion hole, which is formed by the peptidic NH group of Ala 420 and the positive end of a helix dipole moment [Fig. 15(c)]. This oxyanion hole helps to stabilize the ureido (or enolate like) anion of biotin that forms upon decarboxylation.

The structure of the β -subunit of propionyl-CoA carboxylase liganded to both substrates allowed Diacovich *et al.*,⁷⁵ to propose a catalytic mechanism for the enzyme that by inference is applicable to other carboxyltransferase subunits (Fig. 16). The first step in the mechanism involves the decarboxylation of carboxybiotin to form CO₂ and the ureido anion (enolate-like) of biotin, which is stabilized by an oxyanion hole. The ureido anion then acts as a base to abstract the proton from propionyl-CoA to generate an enolate, which is also stabilized by an oxyanion hole. The enolate anion of propionyl-CoA subsequently attacks CO₂ to form methylmalonyl-CoA. It is important to note that this mechanism is very similar to that previously proposed by Wendt *et al.*, for glutaconyl-CoA decarboxylase⁷³ and endorsed by Zhang *et al.*, for yeast acetyl-CoA carboxylase.⁷² Accordingly, for the 12S subunit of the transcarboxylase and the β -subunit of propionyl-CoA carboxylase, it is the ureido anion of biotin that serves as the base required to abstract the proton from the α -carbon of the thioester substrate. In the case of glutaconyl-CoA decarboxylase, biotin serves as the acid to protonate the CO₂ donor. The use of the ureido anion as a catalytic base is in direct contrast to other enzymes in the crotonase superfamily, which utilize either an acidic residue (glutamic or aspartic acid) or a basic residue (histidine or lysine).⁶⁶ It is important to keep in mind that the mechanism presented in Figure 16 represents a starting point for subsequent investigations rather than the final word.

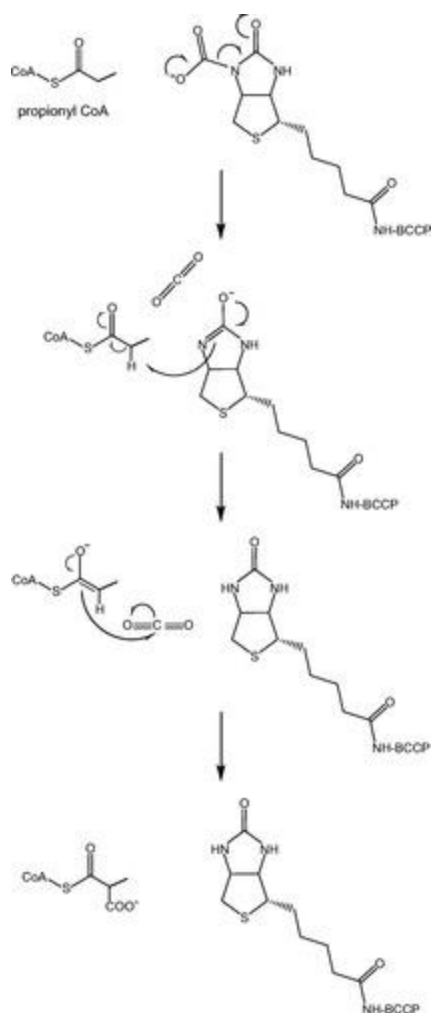


Figure 16 Possible reaction mechanism for propionyl-CoA carboxylase.

Inhibitors of Carboxyltransferase

Fatty acid synthesis plays a key role in both membrane biogenesis and energy storage. As such, the carboxyltransferase of acetyl-CoA carboxylase has been and will continue to serve as a target for herbicides, antibiotics, and anti-obesity agents. In fact, several compounds are already commercially available as herbicides that inhibit the carboxyltransferase component of acetyl-CoA carboxylase.

Plants have two isoforms of the enzyme. The cytosolic isoform is like the eukaryotic form in that it is a single polypeptide with multiple domains corresponding to the two enzymatic activities (biotin carboxylase and carboxyltransferase) and the biotin carboxyl carrier protein. In contrast, the acetyl-CoA carboxylase in chloroplasts in dicots is similar to the bacterial enzyme where the two enzymatic functions and the biotin carboxyl carrier protein reside on separate polypeptide chains. This is in keeping with the hypothesis that chloroplasts evolved from a symbiotic relationship between plants and bacteria. Chloroplasts from grasses have the multidomain form found in the cytosol.

At present, three different classes of herbicides that inhibit the carboxyltransferase domain of chloroplast acetyl-CoA carboxylase from grasses have been identified: the aryloxyphenoxypropionates (e.g., haloxyfop); the cyclohexandiones (e.g., tepraloxym); and the phenylpyrazolines (e.g., pinoxaden). Using the yeast carboxyltransferase domain of acetyl-CoA carboxylase as a model for the plant enzyme, Tong and colleagues determined its structure bound to molecules belonging to each class of herbicide. All of the herbicides bind in

the active site at the interface between the N-terminal domain of one subunit and the C-terminal domain of a neighboring monomer, and all block the acetyl-CoA binding site.⁷⁹⁻⁸¹ Obstructing the acetyl-CoA binding site is consistent with the observed competitive inhibition versus the product malonyl-CoA.⁷² Earlier kinetic analyses with the aryloxyphenoxypropionates and the cyclohexandiones showed noncompetitive inhibition with respect to acetyl-CoA.⁸² All of the crystal structures revealed that the herbicides share three common anchoring points. The first two are the peptidic NH groups of Ala 1627 and Ile 1735 that interact with oxygens on the inhibitors. It is thought that these residues form the oxyanion hole that stabilizes the enolate of acetyl-CoA during catalysis. The third is Leu 1705, which participates in a hydrophobic interaction with the herbicides. The significance of this interaction is underscored by the fact that mutation of Leu 1705 is often observed in plants resistant to the herbicides.⁸¹

In addition to serving as herbicide targets, acetyl-CoA carboxylase has recently been implicated in anti-obesity agents, and molecules that inhibit its carboxyltransferase component are being actively investigated. Humans, like plants, have two isoforms of acetyl-CoA carboxylase. One isoform is located in the cytosol and is required for fatty acid biosynthesis. The other isoform is found in the mitochondria, and it is involved in the regulation of fatty acid oxidation by using the product of the reaction, malonyl-CoA, to inhibit carnitine palmitoyl transferase I. Strikingly, mice, in which the gene encoding the mitochondrial isoform of acetyl-CoA carboxylase has been knocked out demonstrate increased levels of fatty acid oxidation resulting in a lean body mass despite consuming a high fat diet.⁸³ Indeed, the mouse studies provide strong evidence that the mitochondrial isoform of acetyl-CoA carboxylase could serve as an ideal target for anti-obesity agents.

Using high-throughput screening, scientists at Pfizer discovered a molecule (CP-640186) that inhibits both isoforms of mammalian acetyl-CoA carboxylase.⁸⁴ In animal models, CP-640186 decreases fatty acid synthesis while increasing fatty acid oxidation ultimately leading to a reduction in both body fat and weight. Subsequent structural analysis of a complex between the yeast carboxyltransferase domain and CP-640186 showed that the inhibitor binds at the interface of the carboxyltransferase dimer, and it was concluded that this is also the biotin-binding site.⁸⁵ It should be noted, however, that a kinetic analysis of the inhibitor binding versus biotin was not reported, and thus the proposed biotin-binding site is still open to discussion.

Whereas the yeast carboxyltransferase model certainly can be employed as a molecular template for the human enzyme, the fact remains that successful structure-based drug design ultimately requires a detailed understanding of the human enzyme. To this end, scientists at Pfizer, unable to crystallize the human enzyme, mutated nine active site residues in the yeast carboxyltransferase to the corresponding amino acid residues found in the human carboxyltransferase. This variant form of the yeast enzyme was found to bind CP-640186 similarly to that observed in the wild-type yeast carboxyltransferase domain.⁸⁶ The investigators at GlaxoSmithKline were more fortunate in that they were able to crystallize the human carboxyltransferase domain of acetyl-CoA carboxylase in complex with CP-640186 and also found that the inhibitor binds in a similar manner.⁸⁷ More importantly, however, their crystal structure revealed that the human enzyme has three additional α -helices on the C-terminus not present in the yeast carboxyltransferase. Subsequent studies of variant enzymes with the three α -helices deleted showed them to be important for inhibitor binding.⁸⁷ Finally, it should be noted that compounds belonging to the spirochromanones class of molecules, which are structural analogs of CP-640186, were found to inhibit both isoforms of human carboxyltransferase.⁸⁸ Crystal structures of these inhibitors bound to either the carboxyltransferase domain of yeast acetyl-CoA carboxylase or the yeast nine-residue variant version demonstrated that these inhibitors likewise bind in the same site as CP-640186.⁸⁶

Carboxyltransferase Domains without a Crotonase Fold

The carboxyltransferase domains with crotonase folds, as described above, act to carboxylate/decarboxylate thioesters by stabilizing the developing negative charge of the enolate using an oxyanion hole.

Carboxyltransferase domains from a second sub-group of biotin-dependent enzymes, however, do not act on thioester substrates and do not exhibit a crotonase fold. Specifically these enzymes function on keto acid (pyruvate/oxaloacetate) and carbamide (urea) substrates. The enzymes in this sub-group include proteins from all three classes: pyruvate carboxylase (Class I), the α -subunit of the Na⁺ pump oxaloacetate decarboxylase (α -OADC; Class II), the 5S subunit of transcarboxylase (5sTC; Class III), and urea amidolyase (Class I). Phylogenetic analyses of the carboxyltransferase domains of bacterial pyruvate carboxylase and α -OADC indicate that these proteins are evolutionarily distinct from the acyl-CoA carboxylases.⁸⁹ Urea amidolyase is different from all other enzymes of this family and appears to be a recently evolved biotin-dependent enzyme present in only a subset of bacteria, algae, and fungi.^{89,90} Structures of the carboxyltransferase domains of pyruvate carboxylase, α -OADC, 5sTC, and urea amidolyase have been reported, revealing that catalysis in this sub-group does not utilize an oxyanion hole to stabilize either biotin or the keto acid substrate. As such the exact mechanism by which the keto acid enolate is stabilized is unclear at the present time.^{10, 20, 21, 91, 92} It is possible that stabilization of the keto acid enolate is achieved through a combination of metal ion coordination and/or interaction with an Arg/Gln side chain pair. It is also possible that these enzymes stabilize the biotin enolate through tetrahedral coordination of the ureido oxygen.⁹³

Summary

Since that first structure report of a biotin-dependent carboxylase nearly 18 years ago, enormous progress has been made in our understanding of these intriguing enzymes. The goal of this review was not to simply provide descriptions of the biotin carboxylase and carboxyltransferase structures, but rather to critically evaluate what they have and have not revealed about biotin-dependent catalysis. Clearly, there are many unanswered questions, and much research remains. In the end, a thorough understanding of biotin-dependent enzymes is not only important from a basic science perspective but also from a translation approach given that some of these enzymes are targets for the development of anti-obesity agents, antibiotics, and herbicides. Lastly, with the rapidly increasing levels of atmospheric CO₂ there will be a critical need for the development of catalysts that can fix CO₂ to form C—C bonds. The knowledge gained from a thorough understanding of biotin-dependent catalysis will undoubtedly play a key role in the process.

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