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Theoretical Analysis of the Reaction Mechanism of Biotin Carboxylase

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Abstract: A computational approach is taken to clarify the reaction mechanism of biotin carboxylase (BC) by using the B3LYP density functional method. The overall reaction of BC is supposed to consist of two steps: in the first step, carboxyphosphate (CP) is generated from bicarbonate and ATP, and it is subject to nucleophilic attack on its carboxyl group by biotin to form carboxybiotin in the second step. The activation energies for the transition states of the first and second steps are computed to be 46.6 and 7.9 kcal/mol, respectively, demonstrating that the first step limits the overall reaction of BC. In the second step, the ureido moiety of biotin undergoes enolization with the aid of general acid−base catalysis by CP, followed by collapse of CP into CO_2 and phosphate. The resulting bent CO_2 is highly labile and condenses quickly with enolic biotin to give carboxybiotin. Implicit in this scheme as they are, ingenious proton movements between the two substrates, CP and biotin, dictate all of the succeeding chemical events.

Introduction

Biotin-dependent carboxylases play an important role in cellular metabolism by converting such key metabolites as pyruvate and acetyl-CoA to oxalacetate and malonyl-CoA, respectively.[1] The reactions mediated by the respective enzymes, i.e., pyruvate carboxylase (PC, EC 6.4.1.1) and acetyl-CoA carboxylase (ACC, EC 6.4.1.2), comprise two partial reactions: In the first partial reaction protein-bound biotin is carboxylated by biotin carboxylase (BC) with ATP and bicarbonate as cosubstrates (eq 1). In the second partial reaction mediated by carboxyl transferase (CT), the carboxyl group bound temporarily on biotin is then transferred to an acceptor substrate (eq 2). The first partial reaction is common to all the biotin-dependent carboxylases and key to accomplishing the overall carboxylation of acceptor substrates.

$$\text{Enz-Biotin} + HCO_3^- + \text{ATP} \xrightarrow{\text{BC}} \text{Enz-Biotin-}CO_2^- + \text{ADP} + HPO_4^{2-} \quad (1)$$

$$\text{Enz-Biotin-}CO_2^- + \text{acceptor} \xrightarrow{\text{CT}} \text{Enz-Biotin} + \text{acceptor-}CO_2^- \quad (2)$$

Since two of the substrates in the reaction of BC, biotin and bicarbonate, are not labile enough to undergo spontaneous condensation, ATP is needed to activate either or both of the substrates. There are several hypotheses to account for the chemical role of ATP in the reaction of BC, as shown in Scheme 1;[2,3] in mechanism 1 bicarbonate is phosphorylated by ATP to form carboxyphosphate (CP), which is then attacked by the N-1 nitrogen of enzyme-bound biotin.[4] In mechanism 2 the carbonyl group of biotin is phosphorylated first to yield *O*-phosphobiotin, which then undergoes nucleophilic attack on bicarbonate to give carboxybiotin.[5] In light of numerous mechanistic studies,[2,6−14] it would be safe to rule out mechanism 2, and our attention is focused on mechanism 1 as the plausible one for the BC-catalyzed reaction. Nevertheless, since the putative intermediate CP

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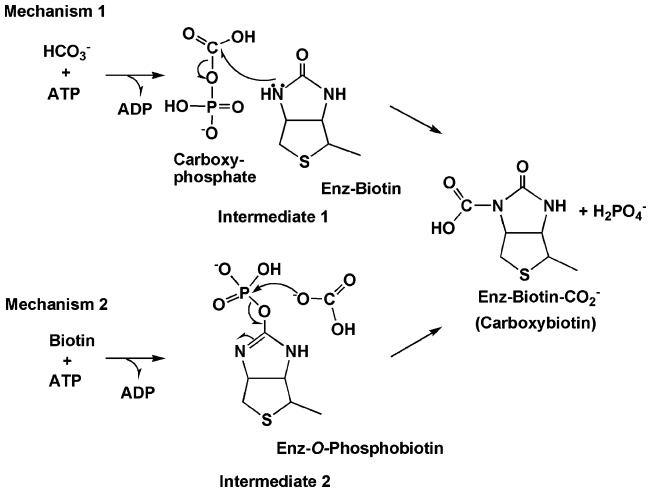
Scheme 1. Mechanistic Possibilities for the ATP-Dependent Carboxylation of Biotin by Bicarbonate, Catalyzed by Biotin Carboxylase (BC)



is either short-lived or accumulated only in quantities insufficient for experimental validation, the definitive evidence for this hypothesis is still lacking.

Another aspect of BC is the fact that it belongs to the ADP-forming ligase family in which most typically a carboxylic acid and an amine undergo condensation in the presence of ATP to form an amide. A relative closest to BC in this group would be carbamoyl-phosphate synthetase (CPS), and in fact they share primary and tertiary structures.[15,16] Nonetheless, they do differ in the substrate to be carboxylated; ammonia in CPS and the urea of biotin in BC. Because of this subtle difference it is not certain whether the two reactions proceed by the same mechanism, despite the fact that the CPS reaction is most likely to proceed via a CP intermediate.[17−21]

These are the rationale behind our theoretical studies of the BC reaction and given the difficulty of proving or disproving the intervention of the intermediate CP in this process experimentally, theoretical treatment would be useful to assess the validity of the proposed mechanisms. In this article, we propose a possible mechanism for the formation of CP and its reaction with biotin using density functional theory (DFT) calculations.

Methods

Computational Methods. The geometries of all stationary points were fully optimized using the B3LYP functional[22,23] with the 6-31G** basis set[24] on the Gaussian 03 software package.[25] The structures and energies of the reactants, intermediates, and products are optimized in the first place, and then the transition state search is carried out on the basis of those structures. All optimized structures were characterized as minima or cols by harmonic vibration analyses. In general, the transition state is defined as the first-order stationary point on a potential energy surface that has a single negative Hessian eigenvalue with an imaginary frequency.[26,27] Therefore, whether a given structure is a transition state or not can be assessed from a calculation of vibrational frequencies. Furthermore, as the reaction goes toward the transition state, the bond involved directly in the

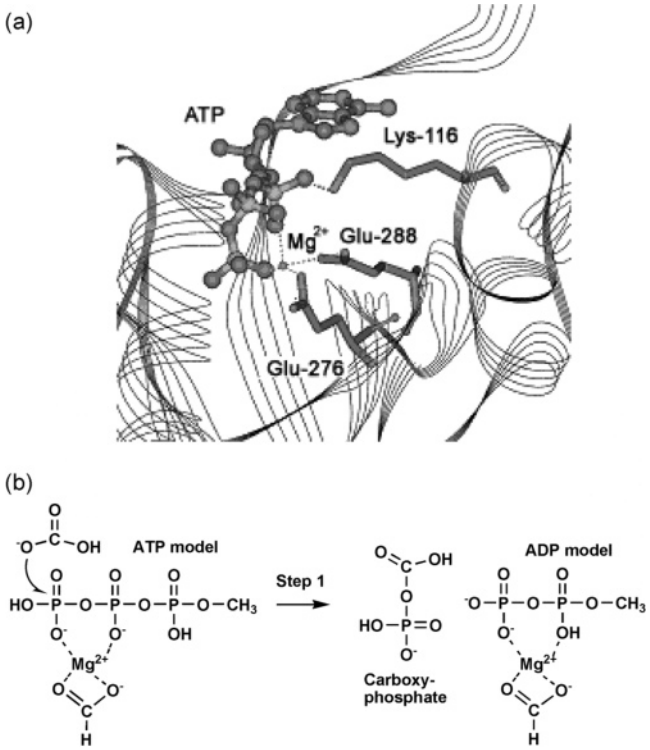


Figure 1. Conformational snapshot of the catalytic site of *Escherichia coli* BC adapted from the structure of mutant E288K (1DV2) by Insight (a) and the ATP model used for the first step of the BC reaction (b).

reaction is elongated with an increase in the amplitude of vibration to give rise to a band at a lower wavenumber.[26,27] Accordingly, the frequency ought to be lower than that in the steady state. Reaction paths were characterized by intrinsic reaction coordinate (IRC) calculations,[28−30] where the initial force constants are provided from the frequency calculations for optimized transition states. IRC was constructed from discrete points at intervals of 0.03 or 0.10 amu$^{1/2}$•bohr. We confirmed changes in geometrical parameters and energies along reaction paths.

Calculation Models. Calculations were performed for mechanism 1 of the BC reaction illustrated in Scheme 1. The crystal structure of a mutant of *Escherichia coli* BC with ATP bound suggests that the side chain of Lys-116 forms a hydrogen bond with the α-phosphate of ATP and that Mg^{2+} ion is coordinated to the side chain of Glu-288.[31] Accordingly, a proton was placed on the α-phosphate of ATP, and Mg^{2+} ion bridging the β- and γ-phosphates is coordinated by formate as a ligand, a mimic of the side chain of Glu-288, as shown in Figure 1. At first, the Mg^{2+}−ATP complex was optimized without formate, but the structure was distorted completely during the optimization, presumably because Mg^{2+} forms a hexacoordinate complex. In addition to these alterations from the BC crystal structure, ATP was modeled by methyl tripolyphosphate to simplify the system and to ease calculations. Biotin has a pentanoic acid side chain, and it was also replaced with a methyl group, as the side chain is not involved directly in the reaction. Furthermore, although the negative charges of ATP are neutralized by Mg^{2+} ion, it was essential to consider protonation of ATP and bicarbonate to overcome the charge repulsion, as both

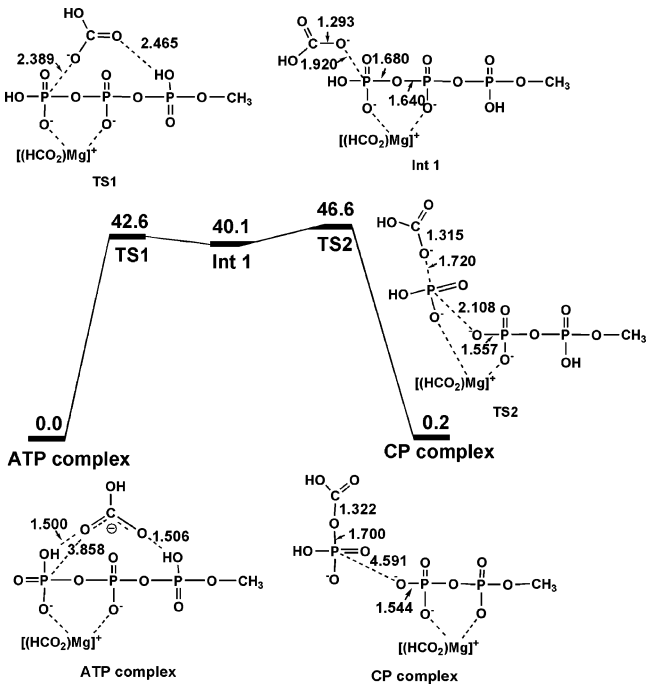


Figure 2. Relative energies and optimized structure computed for the reactant complex, transition states, intermediate, and carboxyphosphate complex for the first partial reaction (step 1) of BC by the B3LYP/6-31G** level of theory. Units are in kcal/mol.

of them are negatively charged. Thus, protons were placed on either or both of the β- and γ-phosphates of ATP and a proton was placed or removed from bicarbonate, but calculations were successful only in the model depicted in Figure 1, where Mg^{2+} is coordinated by the γ-(bidentate) and β-(monodentate) phosphates and formate (bidentate). In addition to this pentacoordinate model, a hexacoordinate complex with another formate or water as ligand was examined, but the energy obtained was not much different.

Results

Carboxylation of biotin is assumed to comprise the following elementary steps: nucleophilic attack of bicarbonate on the γ-phosphate of ATP to form CP and ADP and subsequent reaction of biotin with CP. These two steps were analyzed separately.

Formation of Carboxyphosphate. In light of many experimental results and theoretical considerations of the reactions involving ATP, the nucleophilic attack of bicarbonate on the γ-phosphate of ATP is presumed to proceed by the "inline" mechanism.[4,8−12] An energy diagram calculated for this step and the optimized structures are illustrated in Figure 2. The entire process contains two transition states (**TS1** and **TS2**) and one intermediate (**Int 1**). Initially, one of the oxygen atoms of bicarbonate is located 3.858 Å away from the γ-phosphorus atom of ATP. The distance between these two atoms is shortened to 2.389 Å at **TS1**, and the energy barrier for this state amounts to 42.6 kcal/mol relative to the initial state. The distance is further shortened to 1.920 Å at **Int 1** and eventually to 1.720 Å at **TS2** whose energy level is located 46.6 kcal/mol higher than that of the initial state and 6.5 kcal/mol relative to **Int 1**. By contrast, the P(γ)−O($\beta\gamma$) bond distance is elongated to 1.680 and 2.108 Å at **Int 1** and **TS2**, respectively, and it is eventually cleaved to produce CP and ADP. As a result of this the P(γ)−O(bicarbonate) bond formed is shortened slightly from 1.720 Å at **TS2** to 1.700 Å in the CP complex. The CP complex lies 0.2 kcal/mol above the ATP complex. The imaginary frequency for **TS1** is 89*i* cm^{-1}, and this vibrational motion is related to the stretching of a bond between the bicarbonate oxygen with the γ-phosphorus atom. The imaginary frequency for **TS2** representing an asymmetric stretching vibration of the P(γ)−O($\beta\gamma$) bond being cleaved and the P(γ)−O(bicarbonate) bond forming was evaluated as 122*i* cm^{-1}. Compared with the C−O stretching vibration of anhydrides and the asymmetric stretching vibration of the O−P−O groups in inorganic phosphate and nucleotides of a band at 1100 and 1800−850 cm^{-1}, respectively,[32−36] the results obtained above support the notion that **TS1** and **TS2** are true transition states.

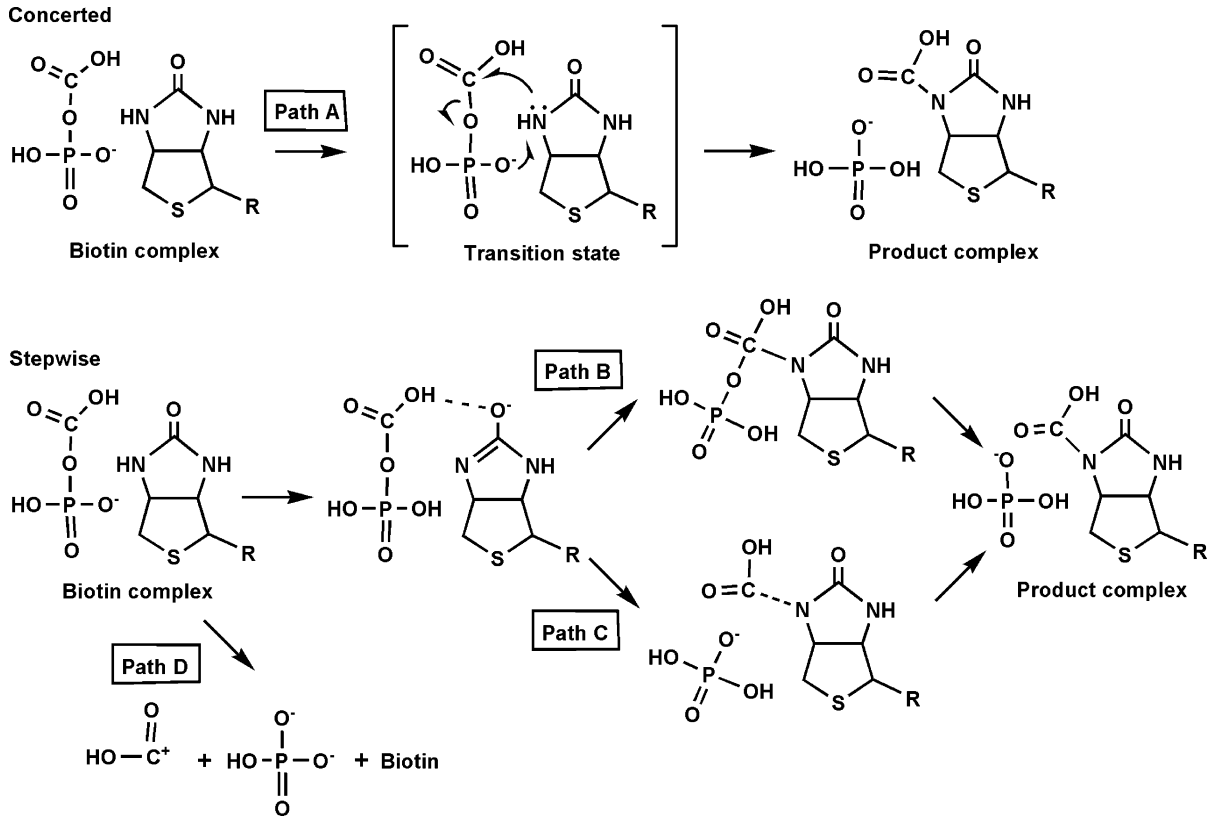
Our calculations indicate that Mg^{2+} ion plays an important role in the reaction of bicarbonate with ATP. As both bicarbonate and ATP are negatively charged, the positive charges of Mg^{2+} ion facilitate the formation of CP by alleviating the electrostatic repulsion between the negative charges.

Mg^{2+}-mediated hydrolysis of a phosphodiester of a hammerhead ribozyme was studied theoretically at the B3LYP/6-31G** level of theory.[37] The hydrolysis reaction is reminiscent of the first step of the BC reaction; the intramolecular nucleophilic attack by an oxygen atom on the phosphorus with charge compensation by Mg^{2+} ion occurs exothermically by only 2.3 kcal/mol with two transition states. The first activation barrier is 18.6 kcal/mol, and the second is 2.2 kcal/mol above the intermediate. The oxygen atom attacks the phosphate from 3.5 Å away in the initial state in this reaction.

Comparison of the first step of the BC reaction with the hydrolysis of the phosphodiester in terms of the type of reaction and the molecular structure involved suggests that the scheme drawn for the first step of BC reaction having two transition states is plausible. Although the energy obtained (46.6 kcal/mol) is fairly high, compared with the 18.6 kcal/mol energy barrier in the hydrolysis of the phosphodiester, the initial state lying nearly at the same energy level as that of the CP complex seems to be reasonable. Since only one bond is reorganized from the phosphoric anhydride O−P−O bond in the ATP complex to a mixed anhydride C−O−P bond in the CP complex, the latter anhydride bond is 2−4 kcal/mol more stable than the former,[38,39] as shown in Figure 2.

Reaction of Carboxyphosphate with Biotin. Four possible pathways were envisaged as plausible mechanisms for the second partial reaction, as shown in Scheme 2. Path A is a concerted mechanism, in which proton abstraction from biotin by CP, nucleophilic attack by one of the ring nitrogen atoms on CP, and the cleavage of the C−O bond in CP occur in a concerted manner. Paths B and C are stepwise reactions, in which abstraction of a proton from biotin by CP occurs first, and then they separate from each other.[14,40,41] In path B, nucleophilic attack of enolic biotin on CP precedes

Scheme 2. Four Viable Reaction Pathways for the BC Reaction



expulsion of phosphate from CP, while in path C, CP collapses to CO_2 first, which then reacts with biotin. In the stepwise mechanisms CP will be more susceptible to nucleophilic attack and the nitride of biotin will serve as a more powerful nucleophile. In path D, spontaneous collapse of CP occurs first to yield the protonated form of carbon dioxide, which must be highly labile toward nucleophiles. Subsequently, proton abstraction and the nucleophilic attack occur in the second step.

Energy diagrams representing each of these paths are depicted in Figure 3. DFT calculations for the concerted pathway (path A) reveal that an energy as much as 35.6 kcal/mol is required to form the product complex via **TS3A**, and it is hence unlikely for the reaction to follow this pathway. Since the spontaneous C−O bond cleavage in path D requires 77.7 kcal/mol, this mechanism should also be ruled out. Thus, it is concluded that nucleophilic attack of biotin on CP should take place via **TS3**, resulting in the formation of **Int 2**. In addition to these four paths the intermediate with a quaternary ureido nitrogen was also examined, but the energy obtained was too high to be optimized by the current level of theory.

In the first stage of the stepwise pathway, which is common to paths B and C, biotin is rendered active as a nucleophile by enolization of its ureido moiety. This is achieved by proton abstraction from the ring nitrogen by CP, and it is manifested in a change in the length of the bonds involved in this process. Thus, the bond length of the C−N and C═O in the urea moiety of biotin is shortened or elongated at **TS3**, respectively, upon the biotin enolization. At the same time, the N−H bond is elongated to 2.438 Å from 1.018 Å at **TS3** because of the proton migration from biotin to CP. The length of the newly formed O−H bond of CP is 0.967 Å, and that of enolic biotin is 1.032 Å at **Int 2**. The optimized structures are shown in Figure 4. At **TS3**, the imaginary frequency representing either the N−H cleaved or the enolic O−H formed is 142*i* cm^{-1}, a value fairly low compared with 3300 cm^{-1} and 3100 cm^{-1} for the stretching motion of O−H groups with an intermolecular hydrogen bond and of N−H groups in hydrogen-bonded secondary amides, respectively.[33,35] Nonetheless, the value may be reasonable, as the IRC with a step size of 0.03 $amu^{1/2}$·bohr connected the biotin complex with **Int 2** via **TS3** smoothly. The activation barrier for **TS3** amounts to 7.9 kcal/mol relative to the biotin complex and **Int 2** lies 3.2 kcal/mol above the biotin complex, as shown in Figure 3. The stepwise mechanism is then separated to paths B and C, which give rise to **TS4B** and **TS4C**, respectively, first.

In path B, the second step begins with an intramolecular proton abstraction from the phosphate of CP by the carboxyl group at **TS4B**. This proton transfer occurs in accord with a difference in the pK_a of phosphates and carboxylic acids, as the pK_a of inorganic and organic phosphates is around 2.0, while that of carboxylic acids is about 3−4[42,43] and it enhances the electrophilicity of the carbonyl carbon of CP, thereby facilitating nucleophilic attack of the enolic biotin to give **Int 3B**. Judging from the frequency for the O−H asymmetric stretching vibration in the steady state, the imaginary frequency of 472*i* cm^{-1} seems to be reasonable for the proton abstraction at **TS4B**. The activation energies are 1.3 kcal/mol for **Int 2** and 4.5 kcal/mol for the biotin complex. **Int 3B** lies 0.9 kcal/mol and 4.1 kcal/mol above **Int 2** and the biotin complex respectively, as shown in Figure 3.

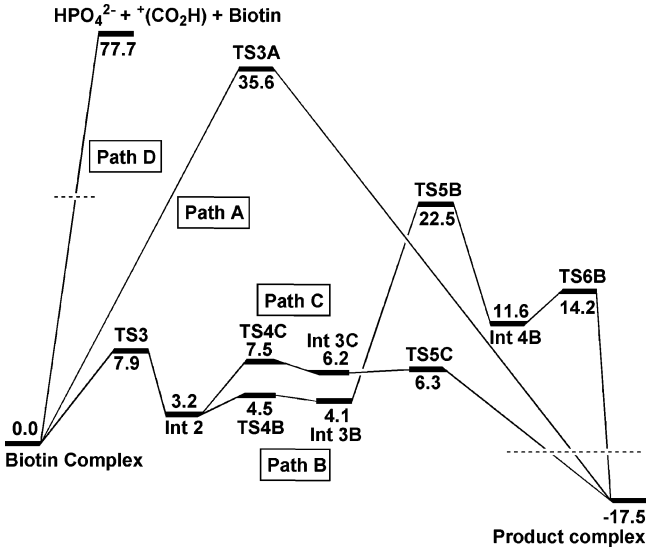


Figure 3. Relative energies computed for the biotin complex, transition states, intermediates, and product complex for the second partial reaction (step 2) of BC by the B3LYP/6-31G** level of theory. Units are in kcal/mol.

In the third stage, the nitrogen of enolic biotin undergoes nucleophilic attack on the carbonyl of CP, concomitant with a proton transfer from the enol to CP to form a tetrahedral intermediate (**TS5B** and **Int 4B**). At the same time, the enolic form of biotin returns to the original keto form. Formation of a tetrahedral intermediate is common in the reaction of carbonyl groups and usually yields the largest activation energy.[44] Hence, the proton transfer is needed to stabilize the nascent oxyanion of a tetrahedral intermediate. The imaginary frequency associated with the asymmetric stretching vibration of the enolic O−H and the C−N bond forming at **TS5B** is 240*i* cm^{-1}, a value regarded as reasonable, as the O−H and C−N stretching vibrations give rise to a band at about 3300 cm^{-1} and about 1000 cm^{-1} in the steady state, respectively.[33,36,45] The activation barrier is 18.4 kcal/mol relative to **Int 3B**, and **Int 4B** lies 7.5 kcal/mol above **Int 3B**.

In the last stage, the C−O bond in CP is gradually elongated and finally cleaved completely upon an intramolecular proton transfer from the carboxyl group of CP to the phosphate. With the expulsion of the phosphate group from the complex, the carbon atom of CP reverts to sp^2 from sp^3. At the end of this stage through **TS6B**, the C−O bond is cleaved completely along with the proton transfer from the carboxyl group to the phosphate. The imaginary frequency representing the asymmetric O−H stretching for the intramolecular proton transfer and the C−O bond cleavage is 395.1*i* cm^{-1} at **TS6B**. The activation barrier amounts to 2.6 kcal/mol relative to **Int 4B**.

In path C, which gives rise to the product complex via the formation of CO_2 and phosphate, CP is split into CO_2 and phosphate spontaneously prior to reaction with biotin (Figure 4). The C−O bond to be cleaved is elongated from 1.426 Å to 1.965 Å at **TS4C** and finally to 2.520 Å at **Int 3C**, where the detached and bent CO_2 is anchored by two hydrogen bonds with the enol of biotin and the detached phosphate. The imaginary frequency associated with the stretching vibration of the C−O to be cleaved is 126*i* cm^{-1} at **TS4C**, a value regarded as reasonable, as the C−O stretching vibrations in anhydrides C−O−C gives rise to a band at about 1100 cm^{-1} in the steady state.[32,33,36] **TS4C** was confirmed by IRC calculations with a step size of 0.10 $amu^{1/2}$·bohr. The activation barrier of **TS4C** is only 3.2 kcal/mol relative to **Int 2**. In other words, the C−O cleavage occurs more easily via **TS4C** than via **TS6B** of path B.

The nitrogen of enolic biotin then undergoes nuclophilic attack on bent CO_2 together with a proton transfer from the enol to CO_2 with a six-membered transition state (**TS5C**). The activation barrier is 0.1 kcal/mol relative to **Int 3C**, and the relative energy is 6.3 kcal/mol from the biotin complex. The much lower activation energy for this pathway stems from the higher electrophilicity of the carbon in question, bent CO_2, than that of CP. In addition, this pathway can avoid formation of a tetrahedral intermediate like **Int 4B**. The imaginary frequency associated with the stretching vibration of the C−N bond forming is 25*i* cm^{-1}. The low frequency may be associated with the low activation energy, and this notion was proven by IRC calculations with a step size smaller than 1.

Discussion

Biotin-dependent carboxylation is intriguing from chemical as well as biological standpoints, as it enables incorporation of bicarbonate into organic substrates under mild conditions to give carboxylic acids. The first partial reaction of BC plays a key role in this context, and its mechanism has been the target of intensive studies.[1−12] The CP hypothesis is the only surviving mechanism for BC, but this intermediate is so elusive that it is difficult to prove its intervention by experiments. As described above, the feasibility of this pathway was scrutinized theoretically for the first time. Carboxylation of biotin was supposed to be accomplished in the two partial reactions: formation of CP from bicarbonate and ATP and its subsequent reaction with biotin. Both of these two steps were simulated successfully with energies of activation of 46.6 and at most 7.9 kcal/mol for the first and second transition states, respectively. The former value may look too large for any reaction to take place smoothly, and there will probably be a path or means to lower this energy of activation. For example, solvent effects and the dielectric constant may be taken into account.[37] Nonetheless, it seems certain that intermediate CP can form by the reaction of bicarbonate with ATP and that the formation of CP is more laborious than its collapse. In other words, the first step limits the overall reaction of BC, and CP will not accumulate in quantities sufficient for detection experimentally at any stage of the reaction.

One of the most prominent features of the second step is that enolization of the ureido portion of biotin is possible. In light of its chemical structure, enolization of biotin is easy to conceive intuitively, and in fact an enol form of biotin has often been postulated in the carboxylation of biotin and subsequent carboxyl transfer from it.[40,46,47] Previous theoretical studies were, however, dismissive of the occurrence of an enolic form, as it is too unstable thermodynamically to form.[48−50] The present study reveals that biotin can undergo

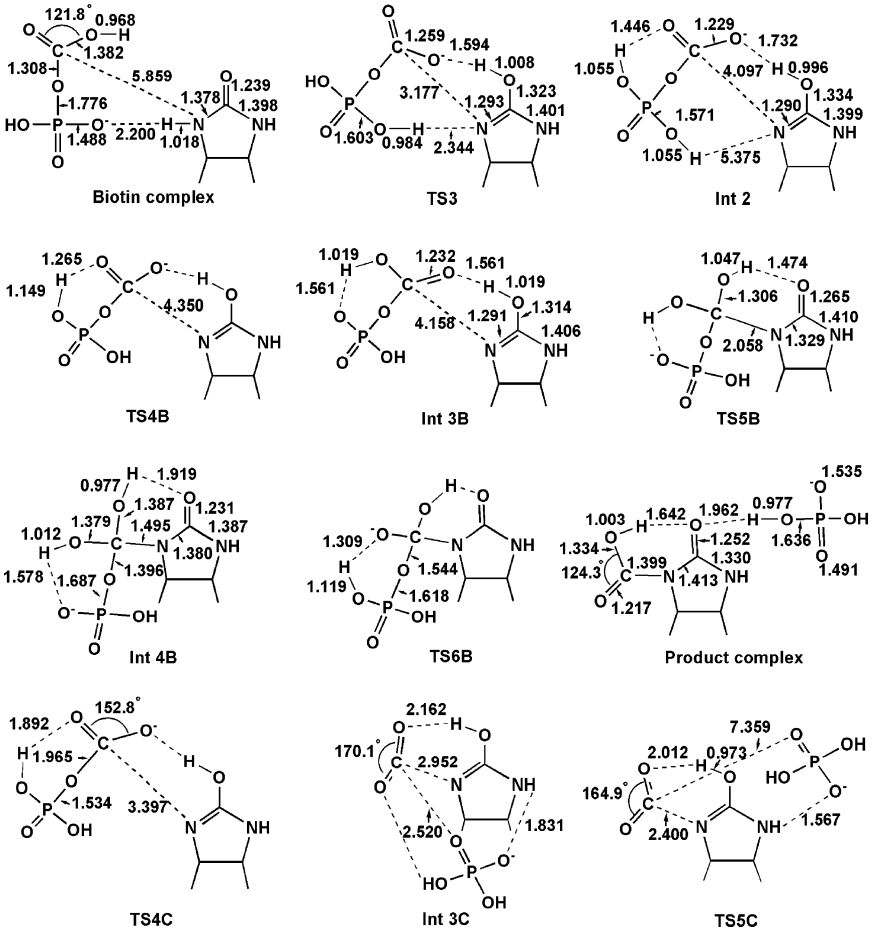


Figure 4. Optimized geometries for the second partial reaction of BC. The tetrahydrothiophene part of biotin is omitted for clarity. Units are in Å.

enolization in the presence of CP with relatively low energy (8 kcal/mol); CP assists this process by serving as a general acid−base catalyst. Thus, the phosphate of CP abstracts a proton from the ureido nitrogen, and the carboxyl group donates a proton to the ureido carbonyl at the transition state (**TS3**). The resulting **Int 2** will follow either one of the two pathways: CP reacts directly with biotin, thereby forming tetrahedral intermediates, or CP generates bent CO_2 which then reacts with enolic biotin. It turned out that the latter pathway is 15 kcal/mol lower in energy, presumably because it can avoid the formation of a tetrahedral intermediate and because bent CO_2 is more electrophilic than CP.

Although the net reaction of the second step is nucleophilic attack of the ureido nitrogen of biotin on the carboxyl group of CP, mobile protons residing initially on the carboxyl and phosphate of CP and on the ureido group play important roles. Thus, proton transfers from CP to biotin and from biotin to CP trigger the entire second step of BC reaction. This kind of behavior is indeed common in many enzymatic reactions.[40,51−58] It is noted that though there are only two components, CP and biotin, in the present system, there are protein and water molecules as well in actual enzymatic systems. In addition, Lewis acids such as Mg^{2+} and Mn^{2+} participate in the carboxylation to polarize the scissile bonds.[59] Some of these components in close contact with substrates at the enzyme active site may reduce the activation energy by handling proton elaborately or acting as the true proton donor or acceptor.[60−64]

Finally, carbamoyl-phosphate synthetase, the closest relative of BC in terms of the type of the chemical reactions they mediate, uses ammonia as the nucleophile in the second step in place of biotin. As ammonia (pK_a 9.3 for ammonium) is a much more powerful base than biotin (pK_a 0.18), it would be advantageous for the carboxylases to use ammonia as a nucleophile. Moreover, enzymes contain a number of potential nucleophiles such as imidazole (pK_a 6.8) which may react with CP equally well or even better than biotin. The advantage of using chemically inert biotin as the nucleophile for CP would then be that it becomes labile through enolization only when it is needed, so that it can avoid unnecessary side reactions. In addition, the product, CB (carboxybiotin), is not the ultimate product; rather the carboxyl group placed temporarily on biotin is transferred eventually to acceptor substrates by the reaction of CT. Although the direction is opposite, this reaction is essentially the same as the BC reaction in that it will involve enolization during the carboxyl transfer.[2,40,41,47,65−68] Hence, it will take place nearly with the same ease as the BC reaction.

Conclusions

We have discussed a possible mechanism of biotin carboxylase (BC) using the B3LYP density functional method. The overall reaction of BC consists of two steps: in the first step,

carboxyphosphate (CP) is generated from bicarbonate and ATP, and it is subject to nucleophilic attack on its carboxyl group by biotin to form carboxybiotin in the second step. Detailed analysis of the potential energy surfaces shows that the first step involves two transition states (**TS1** and **TS2**) and one intermediate. The activation energies for **TS1** and **TS2** were computed to be 42.6 and 46.6 kcal/mol, respectively. In the second step, we considered four possible paths. DFT calculations show that the ureido moiety of biotin undergoes enolization with the aid of general acid−base catalysis by CP, followed by collapse of CP into CO_2 and phosphate. The resulting bent CO_2 is highly labile and condenses quickly with enolic biotin to give carboxybiotin. In the second step the activation energy of the rate-determining step is 10.1 kcal/mol if the reaction traces the stepwise pathway along the most low-lying potential energy surface. Therefore the first transition state in the first step controls the reaction rate in the overall reaction of BC. Our calculations indicate that the structure of biotin is suitable for capturing carbonate from CP.

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Supporting Information Available: Full author list for ref 25; Cartesian coordinates for all optimized geometries; and zero-point energy and thermochemical data at each transition state. This material is available free of charge via the Internet at http://pubs.acs.org.

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