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Chemical and Catalytic Mechanisms of Carboxyl Transfer Reactions in Biotin-Dependent Enzymes

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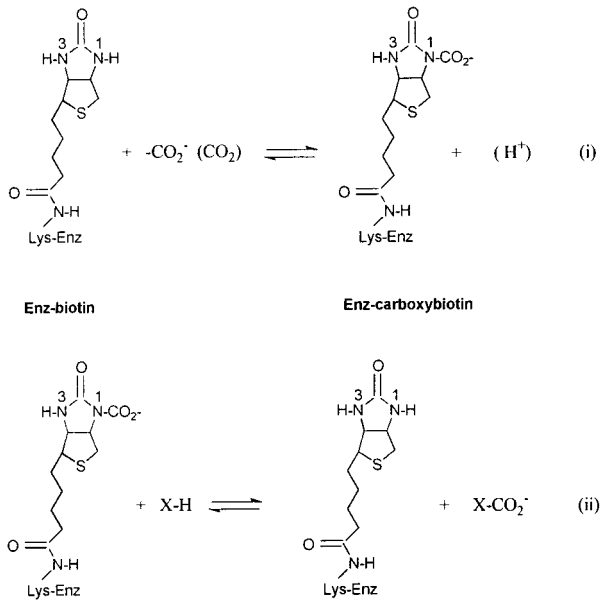
ABSTRACT

Biotin-dependent carboxylases catalyze a variety of carboxyl transfer reactions in a number of metabolic pathways and are found in all free-living organisms. They are large molecules which can comprise a single polypeptide chain with three domains or up to three subunits, each of which performs a particular part of the overall reaction. Biotin plays a central role in the action of these enzymes. In this Account we examine the current state of knowledge of the chemistry of catalysis and consider how the recent explosion of available protein sequence and structural information has assisted our understanding of the mechanisms of biotin-dependent enzymes.

Introduction

Many biological reactions involving carboxyl group transfer are catalyzed by enzymes which contain a covalently bound cofactor, namely biotin (see Scheme 1). These enzymes catalyze three categories of reaction:

Scheme 1



$$HCO_3^- + ATP + RH \overset{Mg^{2+}}{\rightleftharpoons} ADP + Pi + RCO_2^- + H^+ \quad (1)$$

$$RCO_2^- + R'H \rightleftharpoons RH + R'CO_2^- \quad (2)$$

$$RCO_2^- + H_2O + 2Na^+_{intracell} \rightleftharpoons RH + HCO_3^- + 2Na^+_{extracell} \quad (3)$$

The role of biotin is to act as a mobile carboxyl group carrier, transporting the carboxyl group from the site where the carboxyl donor, e.g. HCO_3^-, oxaloacetate, or methylmalonyl CoA, binds to the enzyme to the site where the carboxyl group acceptor, e.g., pyruvate, acetyl CoA, propionyl CoA, or H_2O, binds. This is illustrated in Scheme 1, where the biotin is initially carboxylated on N^1 in reaction i to form carboxybiotin and then transfers the carboxyl group to the acceptor molecule in reaction ii. Biotin-dependent enzymes in general have three domains: a biotin carboxylase domain where reaction i occurs, a transcarboxylase domain where reaction ii occurs, and a biotinyl domain which contains the specific lysine residue to which biotin is covalently bound. Depending on the organism, these domains may occur as separate subunits. For example, in *Methanobacterium thermotrophicum*, pyruvate carboxylase has the biotinyl and transcarboxylase domains on one subunit and the biotin carboxylase on another, whereas in the tetrameric pyruvate carboxylases from some bacteria, yeast, insects, and vertebrates, each subunit has all three domains in a single polypeptide chain.[1] In acetyl CoA carboxylase from

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John Wallace graduated with Honors in agricultural biochemistry in 1960 and received a Ph.D. in 1965, both from Sydney University. He then undertook postdoctoral studies as an 1851 Exhibition Fellow with Nobel Laureate Professor Sir Hans Krebs FRS at Oxford University. His association with pyruvate carboxylase began with its co-discoverer, Professor Merton Utter, at Case-Western Reserve Medical School, Cleveland, Ohio, in 1966 and continued with this enzyme's other co-discoverer, Dr. Bruce Keech, in the Adelaide University Biochemistry Department in 1969, when he returned to Australia as a Queen Elizabeth II Fellow. He joined the Faculty there in 1970, and while continuing to work on pyruvate carboxylase has also investigated the insulin-like growth factor system. He was awarded the LKB Medal of the Australian Biochemical Society in 1986 and was promoted to Reader in Biochemistry in 1987. In 2000, he was awarded the Lemberg Medal by the Australian Society for Biochemistry and Molecular Biology and was promoted to a Personal Chair in Biochemistry.

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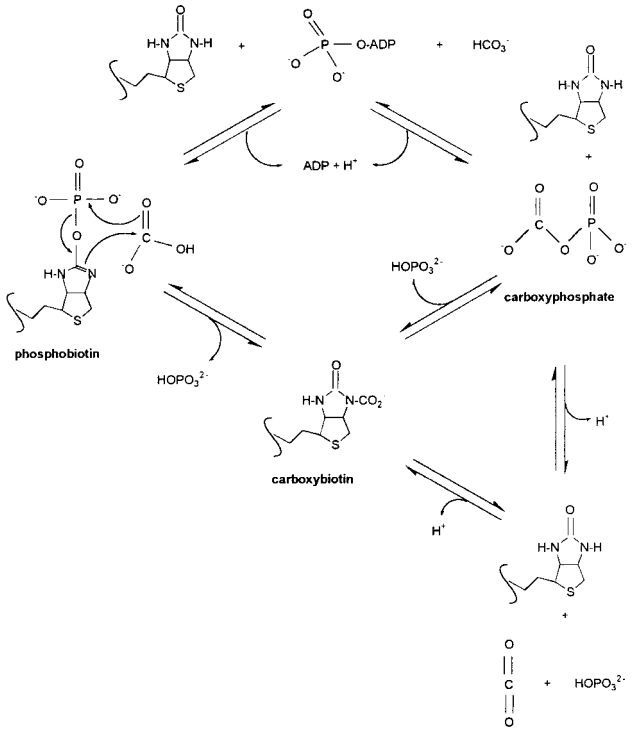
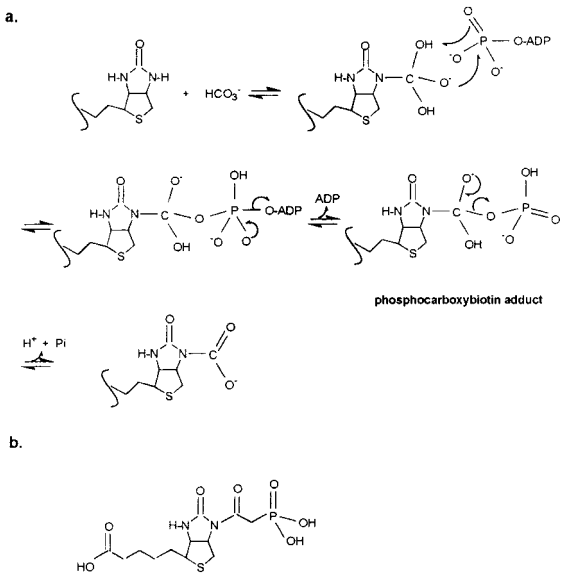
Scheme 2



Scheme 3



Escherichia coli, there are four subunits with the transcarboxylase function split between two.[2]

Since Knowles's review,[3] there have been several reviews on biotin-dependent enzymes.[1,2,4] Here we aim to describe the progress that has been made in understanding the mechanism of action of the biotin-dependent enzymes since 1989. A feature of this progress has been the great increase in the availability of amino acid sequence information and the solving of the structure of biotin carboxylase subunit of *E. coli* acetyl CoA carboxylase[5,6] as well as that of a functionally related enzyme, carbamoyl phosphate synthetase.[7,8]

The Chemical Mechanism of Biotin Carboxylation in Reaction 1

Knowles[3] considered a number of proposed mechanisms for the carboxylation of biotin in reaction 1. These included the initial formation of a phosphobiotin intermediate which then reacts in a concerted cyclic reaction with bicarbonate to form carboxybiotin and Pi (see Scheme 2). Another mechanism involved formation of carboxyphosphate (see Scheme 2), which then directly carboxylates biotin via a nucleophilic attack by the 1′-N of biotin on the carboxyl carbon, or the carboxyphosphate decarboxylates to form the strong electrophile CO_2, which is then the carboxylating species. A third mechanism involved the initial formation of phosphobiotin followed by "in-line" attack on the phosphorus by bicarbonate to form carboxyphosphate, which then carboxylates biotin, either directly or via CO_2. Knowles[3] concluded that the most likely chemical route to the formation of carboxybiotin in reaction 1 was via a carboxyphosphate intermediate formed directly from ATP and bicarbonate. As yet there has not been a direct demonstration of carboxyphosphate by NMR nor conclusively by its isolation,[9,10] as was the case for carbamoyl phosphate synthetase.[11] However, the main evidence on which this conclusion was based was (a) that biotin carboxylase and pyruvate carboxylase can utilize the carboxyphosphate analogue, carbamoyl phosphate, as substrate to phosphorylate ADP[12,13] and (b) that phosphonoacetate, another carboxyphosphate analogue, is an inhibitor of pyruvate carboxylase.[13,14] Furthermore, the stereochemistry of the γ-phosphate group of ATP as it is converted to Pi by pyruvate carboxylase undergoes inversion.[15] This effectively rules out a mechanism in which carboxyphosphate is formed by reaction of bicarbonate with phosphobiotin unless this occurred by an adjacent attack of bicarbonate, which is unprecedented in enzymic chemistry. In the reaction catalyzed by propionyl CoA carboxylase, one ^{18}O from $HC^{18}O_3^-$ ends up in Pi,[16] and this also occurs in a biotin-independent ATPase reaction catalyzed by biotin carboxylase.[17] This strongly suggests a direct reaction between ATP and bicarbonate that does not require biotin.

In 1991, Kluger and Taylor[18] proposed a novel mechanism for the carboxylation of biotin based on model studies showing that the conjugate base of urea will form an adduct with an adjacent carboxylate[19] and model studies of the intramolecular dephosphorylation of the carbonyl hydrate of methylacetoin diethyl phosphate. In this mechanism, biotin first reacts with bicarbonate to form an adduct as shown in Scheme 3a. This adduct then attacks the γ-phosphate of ATP, resulting ultimately in a phosphocarboxy adduct of biotin. Finally, this species undergoes an intramolecular reaction in which Pi is expelled, leaving carboxybiotin. The proposed reaction[18] is chemically feasible and operates well in both directions. In addition, it accounts for the stereochemistry of the reaction of the γ-phosphate of ATP and the incorporation of oxygen from bicarbonate into Pi. Interestingly, Strongin and co-workers[20] recently synthesized an adduct of biotin in which phosphonoacetate was linked to 1′-N (see

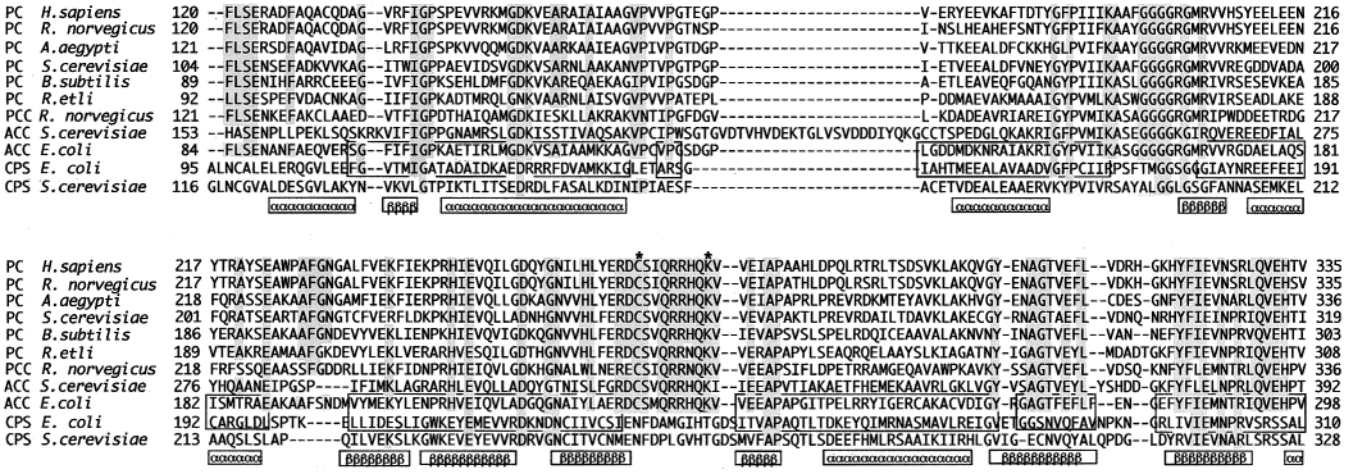


FIGURE 1. Multiple sequence alignment of selected regions of the biotin carboxylation domains from a representative selection of eukaryotic and prokaryotic examples of pyruvate carboxylase (PC), propionyl CoA carboxylase (PCC), and acetyl CoA carboxylase (ACC), shown by ψ-BLAST search to be related, compared with the carboxyphosphate synthetic domain of carbamoyl phosphate synthetase (CPS) of *E. coli* and *S. cerevisiae* using Clustal W. The highly conserved amino acid residues found in different groups of enzymes are shown by shaded boxes. The open boxes represent the residues of ACC and CPS of *E. coli* superimposed using the Homology/Insight program [Molecular Simulations Inc., San Diego, CA] (see Jitrapakdee and Wallace[1]). The putative Cys-Lys ion pair in the biotin-dependent enzymes is indicated by asterisks. Also shown are the α-helices (α) and β-strands (β) observed in the X-ray crystal structure of the biotin carboxylase subunit of *E. coli* ACC. Sources: PC *Homo sapiens* (human), PC *Aedes aegypti* (mosquito), *Saccharomyces cerevisiae* PC1, PC *Bacillus subtilis*, PC *Rhizobium etli*, PCC (propionyl CoA carboxylase α-subunit) rat, ACC *S. cerevisiae*, ACC *E. coli*, CPS *E. coli*, and CPS *S. cerevisiae*.

Scheme 3b), resembling the phosphocarboxy adduct in Scheme 3a. It was shown that this phosphonoacetate adduct was an inhibitor of biotin carboxylase and binds more than 10-fold more tightly to the enzyme than free biotin.[21] Although an apparently feasible mechanism, a consequence of the biotin carboxylation reaction proposed Kluger and Taylor[18] is that biotin is required for ATP cleavage, and this has been shown not to be the case in biotin carboxylase, which catalyzes a bicarbonate-dependent, biotin-independent ATP cleavage reaction.

In support of the intermediacy of carboxyphosphate, other model studies on reactions between arylamines and organic carbonates, which are analogous to that between bicarbonate and biotin, have shown that the synthesis of N-aryl carbamate esters is catalyzed by phosphorous acids, via formation of a phosphocarbonate species which is analogous to carboxyphosphate.[22]

There are similarities between biotin carboxylation in reaction 1 and the formation of carbamate in the carbamoyl phosphate synthetase reaction, in which carboxyphosphate has been shown to be an intermediate.[11,23] In addition, there are amino acid sequence homologies between the biotin carboxylase domains of biotin-dependent enzymes and the carboxyphosphate domain of carbamoyl phosphate synthetase[1,3,24,25] (see Figure 1) and structural similarities between this domain and biotin carboxylase[1,5−7,26,27] (see Figures 1 and 2). Figure 1 shows that, while there is a large degree of sequence identity between parts of the sequences of pyruvate carboxylases from a wide range of organisms, rat propionyl CoA carboxylase and biotin carboxylase subunits of acetyl CoA carboxylase from *E. coli* and yeast, this extends to a much lesser extent to the large subunit of carbamoyl phosphate synthetase. Nevertheless, as indicated by the open boxes, there are considerable regions of structural homology

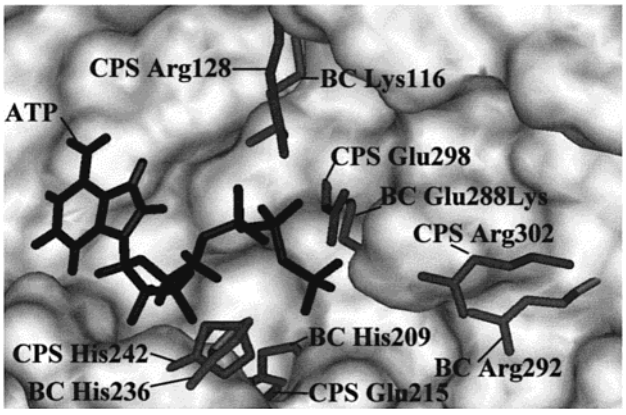
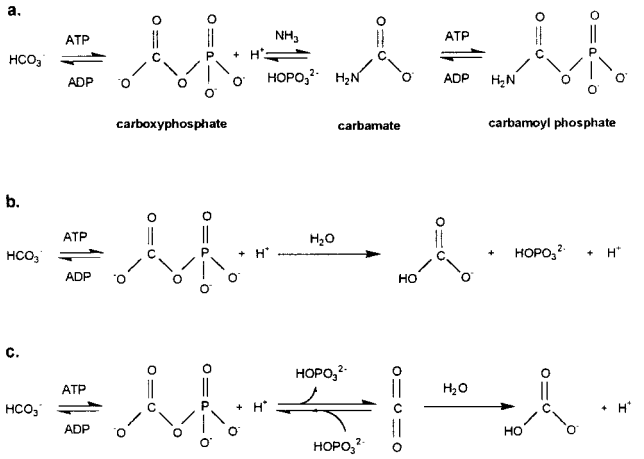


FIGURE 2. Molecular surface representation of part of the superimposed active sites of the biotin carboxylase (BC) subunit of *E. coli* acetyl CoA carboxylase and the carboxyphosphate domain of the large subunit of *E. coli* carbamoyl phosphate synthetase (CPS). Shown in stick form are the side chains of some of the amino acid residues demonstrated to be involved in the binding of ATP, HCO_3^-, or metal ions and/or in catalysis, as described in the text. The structures of the inactive mutant [Glu288Lys]-BC with ATP bound[5] (PDB 1DV2) and of CPS with AMPPNP bound[8] (PDB 1BXR) were superimposed via the common atoms of the purine rings using InsightII software (version 98.0, MSI, San Diego).

between the N-terminal half of the large subunit of carbamoyl phosphate synthetase and biotin carboxylase from *E. coli*.[1] Thoden and co-workers[6,7] have also performed extensive structural comparisons between these two proteins, and, although the sequence identity is only 24% with 47% similarity,[7] they also found regions of strong structural similarity with rms deviations of between 1.6 and 2 Å between structurally equivalent α-carbons in the A, B, and C domains, over a total of 276 residues. This structural similarity also extends to the side chains of catalytically important amino acids in the two proteins,

Scheme 4



some of which are shown in Figure 2. All of the carbamoyl phosphate synthetase residues shown in Figure 2 have been shown to be involved in the binding of ATP, HCO_3^-, or metal ions and/or in catalysis, by site-directed mutagenesis[7] or from the structure of the enzyme.[6,7] The residues in Figure 2 align across all the enzymes in the sequence alignments shown in Figure 1, with the exception of BC His236 and CPS His242, where there is a slight staggering of sequence alignments between the biotin-dependent enzymes and the carbamoyl phosphate synthetases.

The reaction catalyzed by carbamoyl phosphate synthetase is shown in Scheme 4a. After formation of carboxyphosphate, ammonia (derived from glutamine) is carboxylated to form carbamate, analogous to the carboxylation of the 1′-N of biotin. Another ATP then phosphorylates the carbamate to form carbamoyl phosphate. In the mechanism of biotin carboxylation in reaction 1, it is uncertain whether carboxyphosphate or CO_2 is the carboxylating species (see Scheme 2). Gibson et al.[28] have studied the ATPase reaction catalyzed by carbamoyl phosphate synthetase in the absence of glutamine/ammonia and shown that the initial rate of bicarbonate-dependent ATP cleavage was faster than the nonenzymic rate of CO_2 hydration. If in the carbamoyl phosphate synthetase reaction pathway carboxyphosphate is stabilized until it is attacked by ammonia, then the ATPase reaction could be expected to occur as shown in Scheme 4b, with rapid and concurrent ATP cleavage and proton release. If, however, carboxyphosphate was induced to decarboxylate to form CO_2 as a carboxylating species, then the reaction would occur as shown in Scheme 4c, with the slower non-enzyme-catalyzed hydration of CO_2 resulting in proton release lagging behind the rapid ATP cleavage under the conditions of the experiment.[28] The former was found to be the case, indicating that the reaction proceeds as in Scheme 4b.[28] No NMR signal corresponding to $^{13}CO_2$ was detected when $H^{13}CO_3^-$ was used as a substrate.[28] The underlying assumption in these experiments is that if carboxyphosphate decarboxylates to form CO_2 and Pi, the CO_2 is then hydrated in a reaction not catalyzed in any way by the enzyme. However, the reaction being studied was an abortive partial reaction, running at about 15% of the rate of the overall reaction. In the absence of the ammonia substrate, water may be able to enter the active site and hydrate CO_2, in a reaction that occurs more rapidly than in the bulk solvent. In this case, there would not be sufficient accumulation of CO_2 to be detectable by NMR, and it would not be possible to distinguish between the two proposed mechanisms of carboxyphosphate hydrolysis on the basis of the kinetics of proton release.

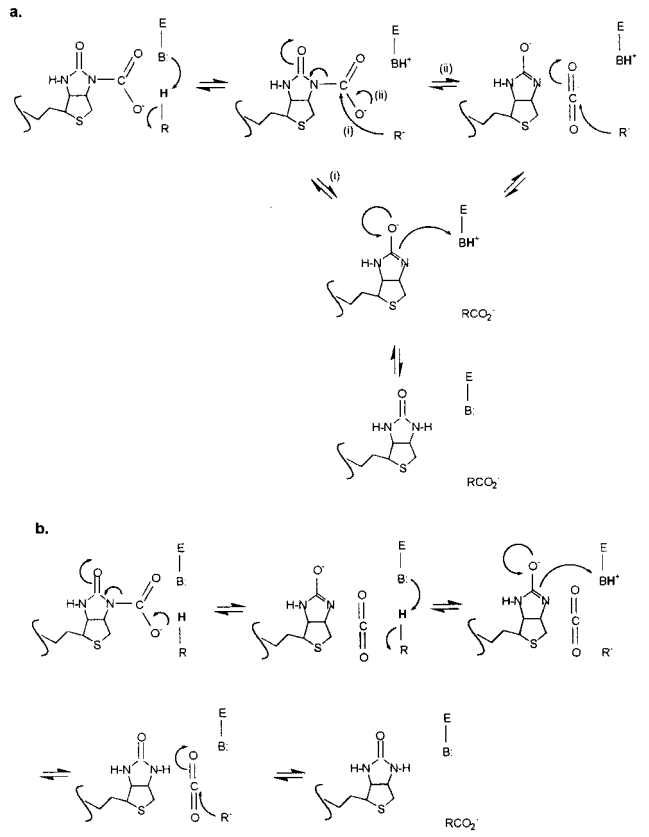
Finally, Gibson et al.[28] failed to observe washout of ^{18}O from [γ-^{18}O]ATP, which would have been expected to occur if CO_2 was formed from carboxyphosphate. However, as recognized by the authors, this washout would occur only if the formation of Pi and CO_2 from carboxyphosphate was reversible and if the Pi was able to rotate freely in the active site. Similar results were obtained with biotin carboxylase, but $^{13}(V/K)$ isotope effects suggested that the forward commitment of the reaction of carboxyphosphate was large.[29]

The final aspect of biotin carboxylation in reaction 1 to be considered is the order of proton and carboxyl group transfers. In transcarboxylase and the pyruvate carboxylation reaction of pyruvate carboxylase, there appear to be stepwise proton and carboxy group transfers (see below). In terms of reaction symmetry and conservation of mechanism, the favored route to carboxylation of biotin from carboxyphosphate in reaction 1 also involves initial proton abstraction (probably by an enzymic base, see below) from the 1′-N of biotin, resulting in the formation of the enolate, which can nucleophilically attack the carboxylating species. However, a recent theoretical study has raised the possibility of there being concerted carboxylation and deprotonation of biotin in this reaction and, at the same time, proposed a long sought-after mechanistic role for the sulfur of biotin.[30] Grant[30] performed ab initio calculations on a biotin derivative in which the pentanoic acid group was replaced with a methyl group. Grant[30] has calculated that a twisted conformation of the bicyclic ring structure can lead to orbital overlap between the sulfur and the ureido group and hence pyrimidalization of the 1′-N, with a consequent increase in its nucleophilicity. The twisted conformation is proposed to mimic an enzyme-stabilized transition state in which carboxylation of 1′-N and deprotonation might occur at the same time. A piece of experimental evidence that might support this proposal is the finding of Tipton and Cleland[29] that the $^{13}(V/K)$ isotope effect for biotin carboxylation by biotin carboxylase increased when the reaction was run in D_2O. This suggests concerted proton and carboxyl transfers; however, as these authors point out, the effect may also be caused by concerted deprotonation of the carboxyl group and decarboxylation of carboxyphosphate.

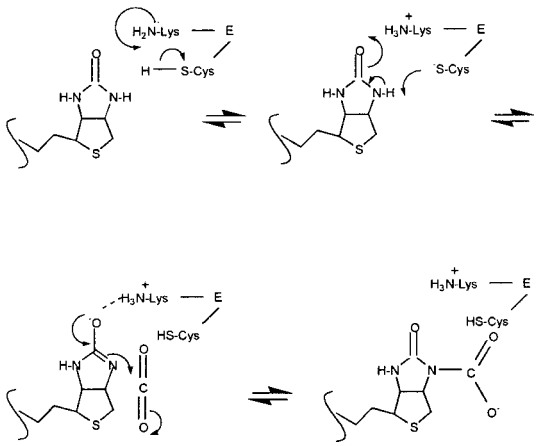
Other Carboxyl Transfer Reactions

The carboxylation of biotin by carboxylated substrates in reactions 2 and 3 and the transfer of carboxyl groups from carboxybiotin to acceptor substrates in reactions 1 and 2

Scheme 5



Scheme 6



also involves carboxyl group and proton transfers between biotin and the substrate (see Scheme 5).

Model studies on the decarboxylation of carboxybiotin[31] and *N*-carboxy-2-imidazolidinone[32] suggest that, at physiological pH's, decarboxylation of carboxybiotin follows a pathway in which CO_2 and the enolate of biotin are formed, suggesting that stepwise carboxyl and proton transfers occur in biotin-dependent enzymes. As reported by Knowles,[3] carboxyl and proton transfers are stepwise in both transcarboxylase and pyruvate carboxylase. In pyruvate carboxylase proton transfer steps flank a central carboxyl transfer (Scheme 5a) and not vice versa (Scheme 5b).

Catalysis

(i) Metal Ions. Biotin-dependent enzymes which catalyze reaction 1 have a requirement for a free divalent metal ion, in excess of that required for complexation in MgATP. Carbamoyl phosphate synthetase also has a requirement for Mg^{2+}, and recently an X-ray crystallographic structure of the *E. coli* enzyme complexed with ADP, Pi, and Mn^{2+} has been published[5] (N.B.: the enzyme is fully active with either Mn^{2+} or Mg^{2+}). The structure of the carboxyphosphate synthetic domain of the large subunit shows two Mn^{2+} ions bound, both of which are apparently ligated by the phosphoryl oxygens of both Pi and the β-phosphate of ADP. One of the Mn^{2+} ions is also coordinated to the side-chain oxygens of Gln285 and Glu299, while the other is coordinated to side-chain oxygens of Asn301 and Glu299.[5] Amino acids corresponding to Glu299 and Asn301 are highly conserved not only across carbamoyl phosphate synthetases from other species, but also across biotin-dependent carboxylases, while Gln285 is replaced by Glu in the biotin-dependent enzymes (see Figure 1). This suggests similar binding sites for Mg^{2+} in the biotin-dependent enzymes. Recently, two equivalents of VO^{2+} were shown by EPR to bind at the biotin carboxylation site of pyruvate carboxylase, one involved in nucleotide binding and the other having a strong interaction with HCO_3^-.[33] This suggests that metal ions may be involved in positioning the γ-phosphate of ATP and HCO_3^- for carboxyphosphate formation and reducing charge repulsion between these two negatively charged species, as well as stabilizing the developing negative charge on the γ-phosphate oxygens in the transition state. ATP binds to pyruvate carboxylase in the absence of Mg^{2+} and but less tightly than MgATP.[30] Thus, one role of the Mg^{2+} complexed in MgATP would seem to be to enhance nucleotide binding. Several studies on pyruvate carboxylase have shown that the second Mg^{2+} enhances the binding of biotin (and carboxybiotin) to the site of the biotin carboxylation reaction.[35−38]

(ii) Enzyme Catalysis. From a variety of kinetic studies, it has been proposed that in the biotin carboxylation step of reaction 1, a Cys-Lys ion pair formed the base and conjugate acid involved in the enolization of biotin.[4] The Lys would remove the proton from the Cys to form the thiolate anion, which would act as the base to remove the 1′-N proton from biotin, while the positively charged ϵ-NH_3^+ of the Lys stabilizes the enolate oxygen of biotin (see Scheme 6). This would result in the enolate of biotin containing a very nucleophilic 1′-N, ready to nucleophilically attack the carboxylating species (CO_2 or carboxyphosphate). Werneberg and Ash[40] showed that reaction of *O*-phthalaldehyde with pyruvate carboxylase resulted in the formation of two isoindole derivatives per mole of enzyme active sites. *O*-Phthalaldehyde will form an isoindole derivative between the sulfur of Cys and the ϵ-NH_2 of Lys if they are separated by about 3 Å, as might occur in an ion pair. The effects of the derivatizations on the reactions steps of the pyruvate carboxylase reaction indicated that there was one putative Cys-Lys ion pair at the site of the biotin carboxylation reaction, and the other

was at the site of the pyruvate carboxylation reaction.[40] Two highly conserved Cys residues and five Lys residues were found in the biotin carboxylation domains of a number of biotin-dependent enzymes, including biotin carboxylase. As shown in Figure 1, a conserved amino acid sequence corresponding to that between residues 228 and 239 of biotin carboxylase was found, which contains a Cys and a Lys that are completely conserved across the biotin-dependent enzymes. The shortest distance between any pair of conserved Lys and Cys residues in biotin carboxylase[5] is between Cys230 and Lys238 at 4.2 Å, and bond rotations easily bring the sulfur and ϵ-NH_2 within 3 Å. In addition, it was noted[5] that the ϵ-NH_2 of Lys238 appeared to interact with the ureido oxygen of biotin bound at the active site of biotin carboxylase, as would be predicted if this residue was acting in an ion pair to facilitate the enolization of biotin. Recently, the hypothesis that Cys230 and Lys238 in biotin carboxylase act to enolize biotin has been tested by experiments in which these residues have been mutated in recombinant biotin carboxylase.[41,42] Mutation of Lys238 resulted in a large increase in the K_m for ATP in both studies, suggesting that Lys238 is involved in ATP binding, and this is supported by the finding that Lys238 is selectively modified by the affinity reagent analogue of ATP, adenosine diphosphopyridoxal.[41] While Kazuta et al.[41] found that mutation of Lys238 reduced V_{max} for the biotin carboxylation reaction by 2−5-fold compared to the wild type, Levert et al.[42] found that mutation of Lys238 abolished the ability of the enzyme to carboxylate biotin. Levert et al.[42] suggested that the small effect on the biotin carboxylation reaction seen by Kazuta et al.[41] was due to contamination with genomic wild-type enzyme. Although mutation of Lys238 abolished biotin carboxylation,[42] there was little effect on k_{cat} for the bicarbonate-dependent, biotin-independent ATP hydrolase reaction of biotin carboxylase.[42] Biotin was still able to stimulate the reaction in which ATP is synthesized from ADP and carbamoyl phosphate in the Lys mutant, indicating that biotin is still able to bind to the enzyme in this mutant form, although with much less affinity, and thus that Lys238 is required in the reaction where biotin is carboxylated.

Mutation of Cys230 to Ala resulted in a 50−80-fold increase in K_m for ATP, with little effect on k_{cat} for the bicarbonate-dependent, biotin-independent ATP hydrolase reaction of biotin carboxylase and retention of the ability to carboxylate,[42] again suggesting a role in ATP binding.

It was partly on the basis of solvent deuterium isotope effect experiments and modification of biotin carboxylase by the sulfydryl reagent, *N*-ethylmaleimide, that the mechanism of biotin carboxylation involving a Cys-Lys ion pair was proposed.[29] However, Levert et al.[42] showed that biotin carboxylase also exhibits inverse solvent deuterium isotope effects in the biotin-independent, bicarbonate-dependent ATP cleavage reaction, suggesting that the deuterium-sensitive step occurs in the reaction involving carboxyphosphate formation and prior to the actual carboxylation of biotin. In addition, Levert et al.[42] showed that it is Lys238 that is modified by *N*-ethylmaleimide and not Cys230 and that the rate of this reaction increased with increasing pH, indicating that the p*K* of Lys238 is equal to or greater than 9.4. Thus, Lys238 would appear to be protonated at the outset of the reaction at physiological pH and would thus not be involved in abstracting a proton from Cys230. The general outcome of this very important study of biotin carboxylase[42] is that it is unlikely that Cys230 and Lys238 act as a pair of acid−base catalysts to remove the 1′-N proton of biotin, though both residues appear to be involved in ATP binding. Lys238 interacts with the γ-phosphate of ATP, and by analogy with an equivalent residue in D-Ala:D-Ala ligase[43] (a member of the ATP-grasp superfamily of enzymes; see below), Levert et al.[42] suggested that Lys238 acts to orient the γ-phosphate of ATP for reaction with bicarbonate. In addition, Lys238 may also position the carboxyphosphate intermediate for further reaction and may also interact with the ureido oxygen of biotin, as originally proposed, assisting both its binding and tautomerization. Given the current lack of evidence supporting the involvement of an enzymic base in the abstraction of the 1′-N proton from biotin, Levert et al.[42] suggest the economical possibility that one of the oxygens of the phosphate in carboxyphosphate plays this role.

Recently, Lys116, Lys159, His209, and Glu276 (see Figure 1) of biotin carboxylase were selected for mutation on the basis of their structural homology with conserved residues of the superfamily of enzymes referred to as ATP-grasp.[44] These experiments have confirmed the involvement of these residues in ATP binding.[44]

(iii) Biotin. Apart from its involvement in the chemistry of the reactions catalyzed by biotin-dependent enzymes, biotin and the part of the amino acid chain to which it is attached also appear to stimulate reaction steps in which it plays no direct role. Polakis et al.[12] noted that the phosphorylation of ADP by carbamoyl phosphate catalyzed by biotin carboxylase was stimulated in the presence of biotin. Oxamate, an inhibitory analogue of pyruvate, binds at the pyruvate binding site and signals the movement of biotin to that part of the active site of pyruvate carboxylase.[37,39] Attwood and Graneri suggested that the oxamate inhibition of both the phosphorylation of ADP by carbamoyl phosphate[14] and the ATPase[35] reaction catalyzed by pyruvate carboxylase is due to the oxamate-induced movement of biotin away from the site of the ATP cleavage reaction in pyruvate carboxylase. Recently, on the basis of site-directed mutagenesis experiments[44] (see above), it has been suggested that biotin binding to biotin carboxylase reduces the number of nonproductive binding modes of ATP, thereby allowing for more reactive substrate alignments and hence an increased k_{cat}. This could occur via induction of conformational changes on biotin binding to biotin carboxylase, although calculations based on the high K_m for biotin suggest that these are not large.[44]

It is not only biotin itself that interacts with the biotin carboxylase subunit of *E. coli* acetyl CoA carboxylase to stimulate the reactions catalyzed by this part of the enzyme. Polakis et al.[12] showed that the biotin carboxyl

carrier protein (BCCP) was a much better substrate than free biotin and also stimulated the phosphorylation of ADP by carbamoyl phosphate. BCCP is a small protein subunit of *E. coli* acetyl CoA carboxylase of 156 amino acids, to which is covalently attached biotin at Lys122. Blanchard et al.[45] were able to express and biotinylate an 87 amino acid carboxy-terminal fragment of BCCP (BCCP-87). This proved to be a better substrate than free biotin for both the biotin carboxylase and carboxyl transferase subunits of *E. coli* acetyl CoA carboxylase, in terms of both k_{cat} and catalytic efficiency. The increases in k_{cat} values and even larger increases in catalytic efficiency for BCCP-87 as a substrate compared to those of free biotin indicate that the binding of BCCP to both of the other subunits of *E. coli* acetyl CoA carboxylase is much tighter than that of free biotin. In addition, the increases in k_{cat} values with BCCP-87 as a substrate suggest that either the BCCP binding induces conformational changes in the other two subunits of the enzyme, which enhance catalysis, or that amino acid residues of BCCP themselves participate in catalysis, or a combination of both occurs. This suggests that, in biotin-dependent enzymes where two or more domains are on a single polypeptide chain, substantial interdomain interactions occur.

Future Directions

A detailed understanding of the overall reaction mechanism of carboxyl transfer by the biotin-dependent enzymes, and indeed of the stimulatory roles of various effectors on this multistep process, awaits high-resolution crystallographic structures of the biotin carboxylase subunit of *E. coli* acetyl CoA carboxylase co-crystallized with BCCP or BCCP-87, or of an enzyme such as pyruvate carboxylase, which has all three domains in a single polypeptide. These structural data, combined with the ability to express and characterize site-specific mutant forms mimicking mutations that occur naturally in humans, should advance our understanding of the molecular defects that underlie these genetic disorders.

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