Carboxylations and Decarboxylations

34

Except in science fiction, all life as we know it today is based on carbon chemistry. Living beings either assimilate the required carbon from already made organic compounds (most heterotrophs) or fix inorganic carbon dioxide to produce the organic compounds (most autotrophs). Carbon dioxide is the end product of respiration in all domains of life. Plants, algae, and cyanobacteria fix CO_2 during photosynthesis, while some others do it by using inorganic compounds (lithoautotrophs) (Berg et al. 2010; Berg 2011). In this sense carbon dioxide is the substrate (for carboxylation) or the product (of decarboxylation) of a large number of enzymatic reactions in biology. A carboxylating enzyme usually links either CO_2 or HCO_3^- with an organic acceptor molecule. Enzymatic carboxylations are physiologically significant routes for CO_2 assimilation. And the reverse of carboxylation is decarboxylation. Carboxylations and decarboxylations are an important class of enzymatic reactions that make and break carbon–carbon bonds.

34.1 Reactions and Reactivity of CO_2

Carbon dioxide (CO_2) represents only 0.036% of the atmospheric gases and is able to diffuse through cell membranes. Its hydration product bicarbonate (HCO_3^-), however, cannot. Solubility of carbon dioxide in water, and at physiological pH, is low. It promptly gets hydrated, and the equilibrium favors the bicarbonate form with an apparent pKa (for $[HCO_3^-/CO_2]$) of 6.3. Under slightly alkaline conditions, $[HCO_3^-]$ is therefore much higher than that of dissolved $[CO_2]$]; this makes usage of HCO_3^- advantageous. In fact this is one reason why a decarboxylation event (enzymatic or not) often becomes an irreversible step of a reaction. By the same token, carboxylations are uphill reactions (endergonic) and require input of energy – with HCO_3^- in particular. No wonder that HCO_3^- is activated by phosphorylation to its mixed anhydride carboxylphosphate (see Biotin-dependent carboxylases and Fig. 34.2 later).

N. S. Punekar, *ENZYMES: Catalysis, Kinetics and Mechanisms*,
https://doi.org/10.1007/978-981-13-0785-0_34

The reversible hydration of carbon dioxide to bicarbonate is spontaneous but slow (takes several seconds). It can be accelerated by the ubiquitous enzyme carbonic anhydrase (EC 4.2.1.1; typically a Zn^{2+}-metalloenzyme):

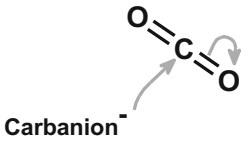
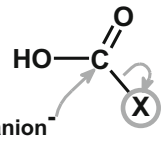
$$\mathbf{CO_2 + H_2O \rightleftarrows HCO_3^- + H^+}$$

This enzyme plays a physiologically significant role (as demonstrated in yeast) in generating HCO_3^- for the carboxylation reactions catalyzed by pyruvate carboxylase, acetyl-CoA carboxylase, and carbamyl phosphate synthetase.

Both carbon dioxide (CO_2) and bicarbonate (HCO_3^-) are essential molecules in various physiological processes. The carbon atom of CO_2 is a good electrophile. A nucleophilic (carbanion) species is best suited to attack this carbon. Molecules undergoing carboxylation possess structural features that stabilize a carbanion for this attack. Indeed many carboxylation reactions occur by the attack of a substrate carbanion on to the carbon atom of CO_2. Bicarbonate is not as good an electrophile as CO_2. The HCO_3^- anion must be activated to a more electrophilic species for reaction. This is achieved by metal ion coordination, dehydration at the enzyme active site, or covalent activation. A carboxylase is thus capable of using either CO_2 or HCO_3^-, and not both. Table 34.1 lists a few examples from these two groups of enzymes.

How do we know whether a carboxylase uses CO_2 or HCO_3^- as its substrate? The chemical (nonenzymatic) equilibrium between CO_2 and HCO_3^- in water is reached over several seconds. This time window provides a good kinetic clue. Consider a carboxylase specific for CO_2 as its substrate. When this enzyme reaction is started with CO_2, initial rates are faster. However, with time the effective [CO_2] decreases due to its hydration to HCO_3^-. This in turn leads to a decreased enzymatic rate and a lower steady-state rate is attained. The net result is an observed burst in the

Table 34.1 Carboxylases use either CO_2 or HCO_3^- as substrate

Enzymes[a] acting on	
Carbon dioxide (CO_2)	Bicarbonate (HCO_3^-)
	
Ribulose-1,5-bisphosphate carboxylase-oxygenase (RubisCO)	Carbamyl phosphate synthetase
PEP carboxykinase	PEP carboxylase
PEP carboxytransphosphorylase	Pyruvate carboxylase
Pyruvate synthase	Acetyl-CoA carboxylase

[a]Each one of them additionally requires cofactors like divalent metal ion, biotin, or thiamine pyrophosphate. Bicarbonate is usually activated to its carbonic–phosphoric mixed anhydride (carboxyphosphate) for reaction; "X" corresponds to phosphate or N-1 of biotin. See specific examples for details

early part of the carboxylase time course. In summary, if a carboxylase shows initial burst kinetics with CO_2, then it uses CO_2 as substrate. For HCO_3^- as the substrate, a lag in the time course is observed. A carboxylase requiring HCO_3^- as substrate behaves exactly opposite – a burst with HCO_3^- and a lag with CO_2 in its kinetics. Careful investigation of lag and burst in carboxylation kinetics is thus a useful tool to distinguish whether that carboxylase uses CO_2 or HCO_3^- as its substrate. We finally note that the lag/burst kinetics should be abolished by including carbonic anhydrase (to rapidly establish $CO_2 \rightleftarrows HCO_3^-$ equilibrium) – to confirm our results.

Carboxylation reactions entail the attack of a suitable carbanion to capture either CO_2 or HCO_3^-. Carboxylases may be classified according to (a) the nature of the attacking carbanion and (b) the cofactor recruited to stabilize the carbanion and/or activate carbon dioxide. Specific mechanistic details available for some well-understood carboxylases are described later. Carboxylation reactions of pyruvate and PEP are central to carbon metabolism and these are discussed next.

34.2 Carboxylation Chemistry with Pyruvate and Phosphoenolpyruvate

Pyruvate is a center piece of carbon metabolism. It is either the substrate or the product in a number of critical reactions (Table 34.2). A large majority of them are carboxylation or decarboxylation reactions.

Pyruvate predominantly exists as a keto acid, while enolpyruvate quickly converts to the keto form in aqueous solution (Fig. 34.1, also see Fig. 29.5, Chap. 29). Enolpyruvate offers a resonance-stabilized carbanion at its C-3 carbon. This carbanionic carbon is where carboxylation of pyruvate (or its phosphorylated enol) occurs. The reaction also requires biotin as a cofactor (see below).

Phosphoenolpyruvate (PEP), the enolase reaction product of glycolysis, participates in a few key biosynthetic steps. PEP is the pyruvoyl donor in two reactions, namely, 5-enolpyruvylshikimate-3-phosphate synthase (of aromatic amino acid biosynthesis) and UDP-N-acetylenolpyruvylglucosamine synthase (of peptidoglycan biosynthesis). But more importantly, PEP is readily carboxylated to oxaloacetate by different anaplerotic enzymes. This carboxylation is invariably accompanied by transfer of the high-energy phosphoryl group of PEP to an acceptor; nature of phosphoryl acceptor may however vary.

The three well-known reactions that carboxylate PEP (box below and Table 34.1) seem to involve enol form of pyruvate as the common reactive intermediate. All of them have a divalent metal ion (Mn^{2+} or Mg^{2+}) requirement. The C-3 carbanion of enolpyruvate attacks either CO_2 (PEP carboxykinase and PEP carboxytransphosphorylase) or activated bicarbonate (PEP carboxylase; enolpyruvate attacks the carbonyl phosphate formed first).

Table 34.2 Metabolic steps involving pyruvate as substrate or product

Reaction (shown as physiologically relevant)	Enzyme; cofactor(s)
Decarboxylations	
Pyruvate → Acetaldehyde + CO_2	Pyruvate decarboxylase; TPP
2 Pyruvate → α-Acetolactate + CO_2	α-Acetolactate synthase; TPP
Pyruvate + CoASH + NAD^+ → Acetyl-CoA + NADH + H^+ + CO_2	Pyruvate dehydrogenase complex;TPP, FAD, lipoate
Pyruvate + O_2 → Acetate + CO_2	Pyruvate oxidase; TPP, FAD
Malate + NAD^+ ⇄ Pyruvate + NADH + H^+ + CO_2	Malic enzyme; Mn^{2+}
Oxaloacetate → Pyruvate + CO_2	Oxaloacetate decarboxylase; Mn^{2+}
Carboxylations	
Pyruvate + HCO_3^- + ATP → Oxaloacetate + ADP + Ⓟ	Pyruvate carboxylase; biotin
Acetyl-CoA + CO_2 + $2e^-$ ⇄ Pyruvate + CoASH	Pyruvate synthase; ferredoxin,TPP
Others	
Pyruvate + CoASH ⇄ Acetyl-CoA + $HCOO^-$	Pyruvate-formate lyase
Pyruvate + NADH + H^+ → Lactate + NAD^+	Lactate dehydrogenase
Pyruvate + L-Glutamate ⇄ L-Alanine +2-oxoglutarate	Glutamate-pyruvate transaminase;PLP
PEP + ADP → Pyruvate + ATP	Pyruvate kinase

Cofactor abbreviations are TPP thiamine pyrophosphate, FAD flavin adenine dinucleotide, and PLP pyridoxal phosphate. Some of these reactions are unique to anaerobic metabolism (pyruvate-formate lyase) and autotrophic carbon fixation mechanisms of archaea (pyruvate synthase and other reactions of reductive TCA cycle)

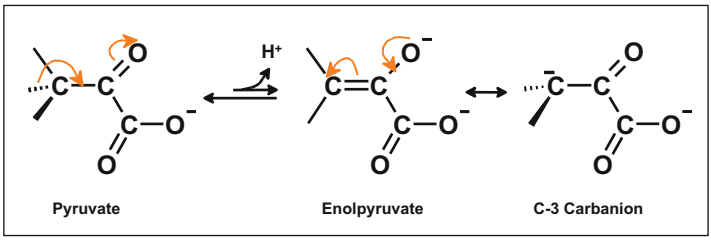


Fig. 34.1 Pyruvate and its enolate. Resonance delocalization of enolate negative charge allows its C-3 carbon atom to react as a carbanion

Enzymes That Carboxylate PEP

PEP carboxylase (from plants and bacteria)

$$\mathbf{PEP + H_2O + HCO_3^- \rightleftarrows Oxaloacetate + Ⓟ}$$

PEP carboxykinase (from fungi, plants, and mammals)

(continued)

$$\mathbf{PEP + GDP + CO_2 \rightleftarrows Oxaloacetate + GTP}$$

PEP carboxytransphosphorylase (*Propionibacterium* sp.)

$$\mathbf{PEP + Ⓟ + CO_2 \rightarrow Oxaloacetate + Ⓟ - Ⓟ}$$

34.3 Cofactor-Assisted Carboxylations

Carboxylation of PEP is facilitated by the associated phosphoryl group transfer. Similarly, all other carboxylations require an input of energy (to activate HCO_3^- in particular) and a suitable cofactor (Table 34.2). Prominent cofactors used in these reactions include biotin, vitamin K, and divalent metal ions. The vitamin K-dependent carboxylase (of prothrombin) is a unique example from blood clotting cascade. The enzyme generates a carbanion on C-4 of the glutamyl residue by abstracting the *proS* hydrogen. This carbanion captures CO_2 to form γ-carboxy-glutamate residues on prothrombin. Autotrophic CO_2 fixation mechanisms of some bacteria (such as pyruvate synthase) are rare examples where thiamine pyrophosphate (TPP) participates in a carboxylation reaction. Here the hydroxyethyl carbanion on TPP (of pyruvate synthase) makes a nucleophilic approach to CO_2. We note here that normally TPP is the cofactor in decarboxylations (discussed later).

Biotin-dependent carboxylases form a physiologically significant group. Ribulose-1,5-bisphosphate carboxylase-oxygenase (RubisCO) is the first and single most important step of photosynthetic CO_2 assimilation. We turn now to these two examples in some detail.

Biotin-Dependent Carboxylases Biotin is a cofactor in many crucial carboxylation reactions. It is therefore an important water-soluble vitamin whose deficiency causes dermatitis. First isolated from egg yolk in 1936, it binds tightly with avidin (a protein from egg white). Avidin–biotin complex is one of the strongest non-covalent interactions known (K_D of 10^{-15} M and a $t_{½}$ of 2.5 years). Most biotin-dependent enzymes are therefore inhibited when incubated with avidin. Biotin is a bicyclic ring with a substituted urea as a functional group for catalytic function (Table 31.2). The cofactor is covalently attached to the ε-amino group of an active site lysine. This charging occurs at the expense of energy (ATP → AMP + Ⓟ-Ⓟ) and is similar to the charging of lipoamide on to transacetylase.

Biotin-dependent carboxylases include enzymes that carboxylate acetyl-CoA, propionyl-CoA, pyruvate, urea, etc. Biocarbonate is the substrate in all these reactions and ATP is required to activate it. One atom of ^{18}O from isotopically

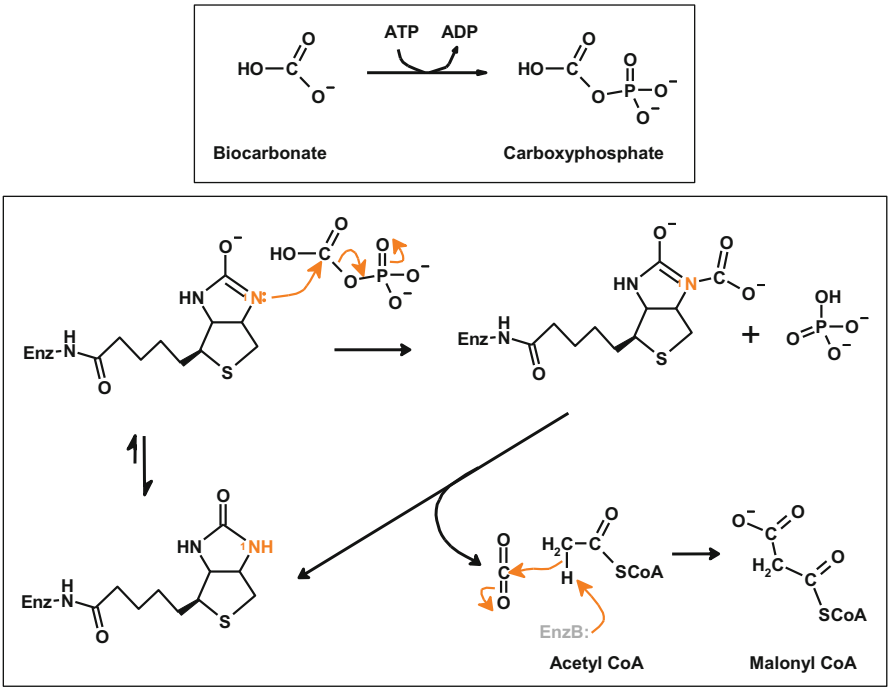


Fig. 34.2 **Biotin-dependent carboxylation of acetyl-CoA**

labeled HCO_3^- ends up in the phosphate formed from ATP (Knowles 1989). This is consistent with the transfer of ATP γ-phosphate to generate *carboxyphosphate* intermediate (Fig. 34.2), at the enzyme active site. It is believed that carboxyphosphate is attacked by N^1 (in its deprotonated state) of biotin to form a carboxy-biotin intermediate. This intermediate (or CO_2 generated from it) is attacked by the substrate carbanion (nucleophile) to form the carboxylated product and regenerate biotin. The entire mechanistic sequence (for acetyl-CoA carboxylase) is shown in Fig. 34.2.

Mechanistic evidence was gathered by a number of elegant isotope exchange experiments and trapping the relevant intermediates. Enzyme-bound carboxy-biotin was trapped by diazomethane and stabilized as its methyl derivative (biotin>N^1-$COOCH_3$). The kinetic and chemical competence of carboxy-biotin was also shown. The carboxylated enzyme was capable of (1) carboxylating the acceptor substrate and (2) synthesizing ATP from ADP and Ⓟ. Their rates were comparable to the overall carboxylation reaction rates. The partial exchanges observed (see box below) support a ping-pong kinetic mechanism. All the biotin-dependent carboxylases studied so far proceed with retention of configuration at the carbon atom being carboxylated.

Exchange Reactions Observed with Acetyl-CoA Carboxylase
Acetyl-CoA carboxylase (and other biotin-dependent carboxylases) is a three-component enzyme:

1. Biotin is covalently bound to the small biotin carboxyl carrier protein (BCCP).
2. Biotin carboxylase charges HCO_3^- on to form carboxy-biotin; it is responsible for the HCO_3^--dependent ADP-ATP exchange.

$$\left[^{14}\mathrm{C}\right]\mathbf{ADP}+\mathbf{ATP}\rightleftarrows\mathbf{ADP}+\left[^{14}\mathrm{C}\right]\mathbf{ATP}$$

3. Carboxyl transferase transfers the active "CO_2" from carboxy-biotin to the acceptor carbanion (acetyl-CoA); this is responsible for the acetyl-CoA-malonyl-CoA exchange.

$$\left[^{14}\mathrm{C}\right]\mathbf{Acetyl\ CoA}+\mathbf{Malonyl\ CoA}\rightleftarrows\mathbf{Acetyl\ CoA}+\left[^{14}\mathrm{C}\right]\mathbf{Malonyl\ CoA}$$

Transcarboxylase is an interesting variation of the biotin-dependent carboxylases. This enzyme (from *Propionibacterium shermanii*) is not a carboxylase but simply transfers the "CO_2" from a donor to an acceptor via the enzyme-bound biotin.

$$\mathbf{Propionyl\ CoA}+\mathbf{Oxaloacetate}\rightleftarrows\mathbf{Pyruvate}+\mathbf{\mathit{S}-Methylmalonyl\ CoA}$$

Transcarboxylase is an example of two-site ping-pong kinetics where the biotin on a flexible swinging arm ferries "CO_2" between two separate active site regions – the donor site and the acceptor site (see Fig. 28.5, Chap. 28).

At least three instances of carboxylation at the N atom may be noted. Carbamyl phosphate synthetase is an example of ammonia carboxylation that occurs without the need for biotin. The ammonia N makes a direct nucleophilic attack on carboxyphosphate (enzyme bound) to form carbamate – which subsequently gets phosphorylated to carbamyl phosphate. However the $-NH_2$ group of urea is normally an unreactive nucleophile. It is thought that such N is deprotonated before its attack on carboxyphosphate. Both the carboxylation at N^1 of biotin and that of urea (by urea carboxylase leading to allophanate) are examples of such N-carboxylations.

RubisCO Ribulose-1,5-bisphosphate carboxylase-oxygenase (abbreviated as RubisCO) is the enzyme responsible for fixing atmospheric CO_2 by green plants and photosynthetic bacteria. RubisCO is the most abundant protein in the world. It neither carboxylates an energy-rich molecule (like PEP) nor uses ATP for activation. RubisCO is one of the abundant proteins localized in the chloroplasts and contains Cu^{2+} and requires Mg^{2+} or Mn^{2+} for its carboxylation activity. Carboxylation of

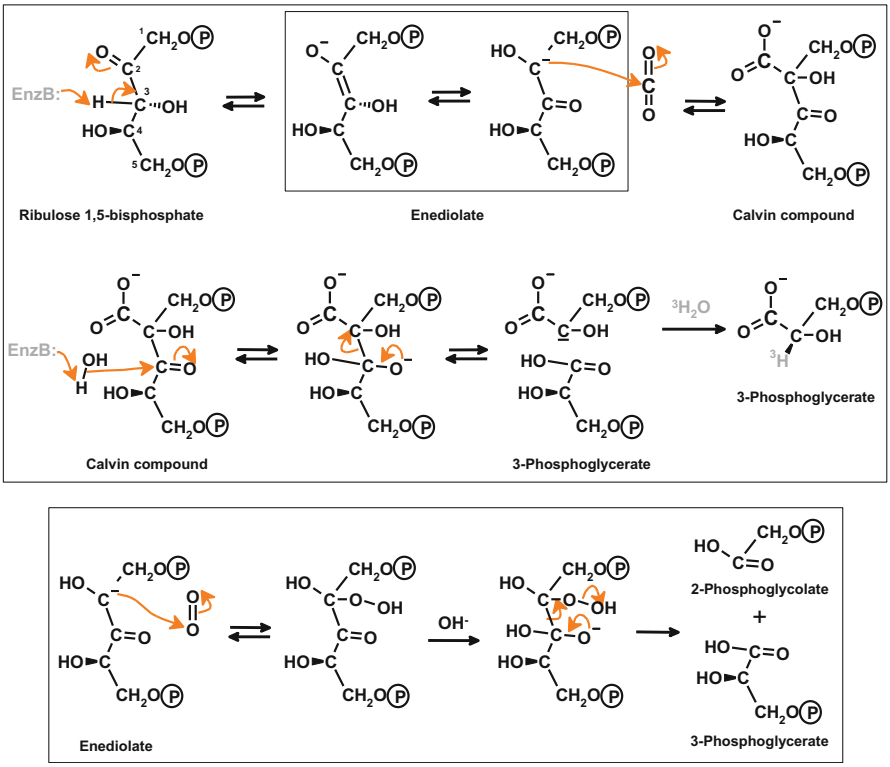


Fig. 34.3 Carboxylation reaction mechanism of ribulose-1,5-bisphosphate carboxylase-oxygenase. The bottom panel shows the oxygenase reaction catalyzed by the same enzyme. The carbon originating from CO_2 is in bold

ribulose-1,5-bisphosphate is associated with its irreversible cleavage to two molecules of 3-phosphoglycerate. This reaction forms the first step of Calvin cycle in plants.

Many labeling experiments have defined the chemical mechanism of RubisCO (Fig. 34.3). These include the following: (a) radioactivity from $^{14}CO_2$ ends up in the C-1 carboxylate of one half of the 3-phosphoglycerate molecules formed, (b) ^3H from the solvent is incorporated at C-2 of the same 3-phosphoglycerate molecule that has got $^{14}CO_2$, and (c) ^3H on the C-3 of ribulose-1,5-bisphosphate rapidly exchanges with solvent protons in the presence of this enzyme. A six carbon intermediate compound (2-carboxy-3-keto derivative of ribulose-1,5-bisphosphate) was postulated by Calvin. Molecules structurally related to this postulated intermediate are excellent reversible inhibitors of RubisCO. We have come across one such inhibitor earlier in 2′-carboxy-D-arabinitol 1,5-bisphosphate (see Fig. 6.9 in Chap. 6).

Table 34.3 Autotrophic CO_2/HCO_3^- assimilation mechanisms

Assimilation pathway[a]	Carboxylases used	Occurrence
Calvin–Benson cycle (reductive pentose phosphate cycle)	RubisCO (and PEP carboxylase)	Plants, algae, cyanobacteria, and many aerobic/facultative aerobic *Proteobacteria*
Reductive citric acid cycle (rTCA cycle)	Pyruvate synthase (Fd), 2-oxoglutarate synthase (Fd), isocitrate dehydrogenase, PEP carboxylase	Green sulfur bacteria, anaerobic or microaerobic bacteria
Reductive acetyl-CoA pathway (requires strict anoxic conditions)	CO Dehydrogenase/acetyl-CoA synthase, pyruvate synthase (Fd), 2-oxoglutarate synthase (Fd)	Prokaryotes like acetogenic bacteria, methanogenic archaea, psychrophiles, hyperthermophiles
3-Hydroxypropionate cycle	Acetyl-CoA/propionyl-CoA carboxylases (biotin)	Green non-sulfur phototrophs
4-Hydroxybutyrate cycles	Acetyl-CoA/propionyl-CoA carboxylases (biotin), pyruvate synthase (Fd), and PEP carboxylase	(Hyper)Thermophilic organisms, *Crenarchaeota*

[a]Depending on the environment and nutritional state, some organisms may display more than one assimilation pathway

RubisCO reaction mechanism begins by proton abstraction from C-3 of ribulose-1,5-bisphosphate (which accounts for tritium exchange with solvent) and enolization of C-2 ketone. The resulting enediol now can display carbanion character at its C-2 and attacks CO_2 to form the carboxylated intermediate (Calvin compound). Nucleophilic attack by water at C-3 and the C-C bond cleavage (between C-2 and C-3) produces two 3-phosphoglycerate molecules. The 3-phosphoglycerate that bears the newly fixed carbon is the one whose C-2 carbanion picks up a solvent proton.

RubisCO is unable to strictly discriminate between CO_2 and O_2 for its substrate (Griffiths 2006). The consequence is the process of photorespiration observed in plants. Apart from its carboxylation reaction, RubisCO is capable of an oxygenase activity. RubisCO functions as a monooxygenase (in the absence of CO_2) and splits ribulose-1,5-bisphosphate into 2-phosphoglycolate and 3-phosphoglycerate. One atom of oxygen from $^{18}O_2$ ends up in the carboxylate group of 2-phosphoglycolate. This supports the mechanism where the enediol (its C-2 carbanion) attacks O_2 (Fig. 34.3).

Autotrophic CO_2/HCO_3^- Assimilation Pathways Reductive pentose phosphate cycle (Calvin–Benson cycle) along with RubisCO is undoubtedly of singular importance in the autotrophic CO_2 fixation in nature. However to date six autotrophic CO_2 fixation mechanisms (Table 34.3) are known. All of them of course have to carry out carboxylation steps either with CO_2 or HCO_3^- or both. The observed diversity reflects the variety of the organisms and the ecological niches existing in nature (Berg 2011).

34.4 Decarboxylation Reactions

Carboxylations and decarboxylations are complementary reactions yet essential components of the global carbon cycle. Mechanistically, decarboxylations are the reverse of carboxylations – but with a difference. While either CO_2 or HCO_3^- is used in a carboxylation step, *decarboxylation invariably results in CO_2 as its product*. It is a different matter however that CO_2 formed quickly gets hydrated (by carbonic anhydrase) to HCO_3^- in the aqueous setting. This makes decarboxylations irreversible and thermodynamically downhill. An organic compound undergoes decarboxylation by losing its carboxylate group by releasing CO_2. Typically this reaction goes through a transition state where a carbanion develops on the carbon atom losing the -COOH group (Fig. 34.4). Decarboxylation rates can be accelerated by stabilizing the developing carbanion. Mechanism(s) to do this include providing a suitable *temporary electron sink*. Such features may be present either within the substrate structure or in an external cofactor recruited for this purpose. We will look at these possibilities in some detail.

Most common organic acids undergoing decarboxylations also contain additional functional groups. For instance, these may be α-amino acids (all the 20 and more), α-keto acids (pyruvate, 2-oxoglutarate, and 2-keto acids of branched chain amino acids), β-keto acids (oxaloacetate and acetoacetate), βγ-unsaturated acids (*cis*-aconitate), or β-hydroxy acids (malate, isocitrate, and 6-phosphogluconate). An incipient carbanion forms during the decarboxylation of all these acids (Fig. 34.4). A β-keto acid substrate offers the possibility of enolization; this in turn can function as a useful temporary electron sink. The β-keto group therefore assists in decarboxylation by delocalizing negative charge. An α-keto group cannot be used in this manner. Both α-amino acids and α-keto acids lack a built-in structural feature to stabilize this developing negative charge. External electron sinks like PLP (α-amino acid decarboxylations; Chap. 35 – Electrophilic Catalysis and Amino Acid Transformations) or TPP (α-keto acid decarboxylations) are required/recruited to help decarboxylate them. While we will deal with these a little later, decarboxylation mechanisms for β-keto acids are discussed first.

β-Keto Acid Decarboxylation Mechanisms The β-keto group provides natural assistance in decarboxylating β-keto acids. This is contingent upon how easily it can be enolized. Low basicity of the β-carbonyl group sets a high-energy barrier and needs further assistance. Nevertheless β-keto group is exploited to support the

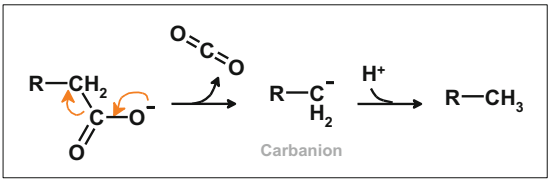


Fig. 34.4 General scheme for decarboxylation of an organic acid and CO_2 release. The product carbanion may be quenched by a proton as shown or by an electrophile

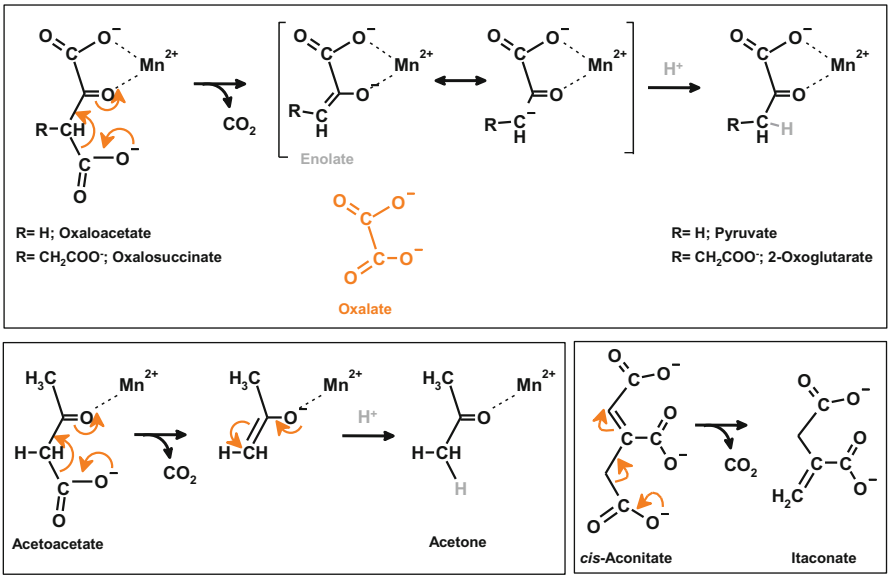


Fig. 34.5 Decarboxylation of β-keto acids. Among the carboxylate groups, the carboxylate that has a suitably positioned β-keto moiety is lost as CO_2. Mn^{2+} functions to accept the developing negative charge. Proposed decarboxylation of *cis*-aconitate by exploiting appropriately located double bond is also shown

developing carbanion in different ways. First, it is made a better electron sink by protonating the developing enolate form. For instance, nonenzymatic decarboxylation of acetoacetic acid is pH dependent and is 50 times faster in its acid form than the corresponding acetoacetate ion. Second, divalent metal ions may assist in stabilizing the negative charge. Mn^{2+} is very well able to accept the developing enolate ion during oxaloacetate decarboxylation (Fig. 34.5; also see Fig. 6.6); indeed oxaloacetate decarboxylase requires Mn^{2+} for activity. Oxalate ($^{-}OOC\text{-}COO^{-}$) mimicking the initial enolate product is an inhibitor of this enzyme. This is in keeping with the proposed oxaloacetate decarboxylase chemical mechanism.

Enzymatic decarboxylation of a β-hydroxy acid shares mechanistic similarities with that of a β-keto acid. The β-hydroxy moiety cannot function as a useful electron sink. Therefore it is converted to a β-keto acid prior to its decarboxylation. Well-characterized examples of this kind include malic enzyme, isocitrate dehydrogenase, and 6-phosphogluconate dehydrogenase. Both malic enzyme and isocitrate dehydrogenase require bound Mn^{2+} for activity. They also require $NAD(P)^{+}$ which accepts the reducing equivalents ($2e^{-}$) and oxidizes the β-hydroxy acid. The two enzyme reactions appear to proceed with the generation of a β-keto acid intermediate. Analysis of kinetic isotope effects, at least with malic enzyme, supports a mechanism where malate oxidation precedes its decarboxylation (see Fig. 27.4,

Chap. 27). Oxalosuccinate is the proposed intermediate of isocitrate dehydrogenase reaction (see box below). Recently a loss of function mutant of isocitrate dehydrogenase was implicated in cancer metabolism – it simply reduces 2-oxoglutarate to 2-hydroxyglutarate (see Chap. 39). Although a β-keto acid intermediate seems chemically reasonable, its actual demonstration can be tricky. It may be tightly bound to the active site, not accessible for trapping reagents or may be ephemeral.

Malic enzyme:

$$\textbf{Malate} \xrightarrow{-2e^-} [\textbf{Oxaloacetate}] \rightarrow \textbf{Pyruvate} + \textbf{CO}_2$$

Isocitrate dehydrogenase:

$$\textbf{Isocitrate} \xrightarrow{-2e^-} [\textbf{Oxalosuccinate}] \rightarrow \textbf{2-Oxoglutarate} + \textbf{CO}_2$$

Oxaloacetate (as also oxalosuccinate) is both an α-keto acid and a β-keto acid. Only the carboxylate that is β to the keto group (previously the hydroxyl group) is lost as CO_2. Interestingly, *cis*-aconitate decarboxylase might exploit the suitably located double bond to facilitate decarboxylation and form itaconate.

Decarboxylation of Acetoacetate As expected for the β-keto acid, both nonenzymatic and enzymatic decarboxylation of acetoacetic acid is promoted by Mn^{2+}. The divalent metal ion acts as a superacid catalyst, polarizes the β-keto group, and makes it a better electron sink. Acetoacetate decarboxylases requiring Mn^{2+} for catalysis (Fig. 34.5) are less common. Instead, there is an efficient and interesting way to use β-keto moiety as an electron sink. This more effective strategy employs a protonated Schiff base form. While it is difficult to protonate the oxygen of β-carbonyl group (poor base), corresponding imine nitrogen (Schiff base) is readily protonated. This *cationic imine* is an excellent sink to stabilize the carbanion formed during decarboxylation. *Formation of imines at adjacent carbonyl groups is a general mechanism for catalysis when carbanions are generated during the reaction.* No wonder that amines are effective catalysts in decarboxylating β-keto acids. For instance, aniline (pKa of 4.8) can accelerate acetoacetate (pKa of 3.7) decarboxylation rate maximally at pH 4.2. The "aniline–acetoacetate complex" breaks down much faster than the acid alone. A well-documented enzyme example is acetoacetate decarboxylase from *Clostridium acetobutylicum*. In its reaction mechanism (Fig. 34.6), active site lysine $–NH_2$ forms a Schiff base with acetoacetate. This cationic imine acts as an electron sink to stabilize the carbanion formed at C-2 in the transition state.

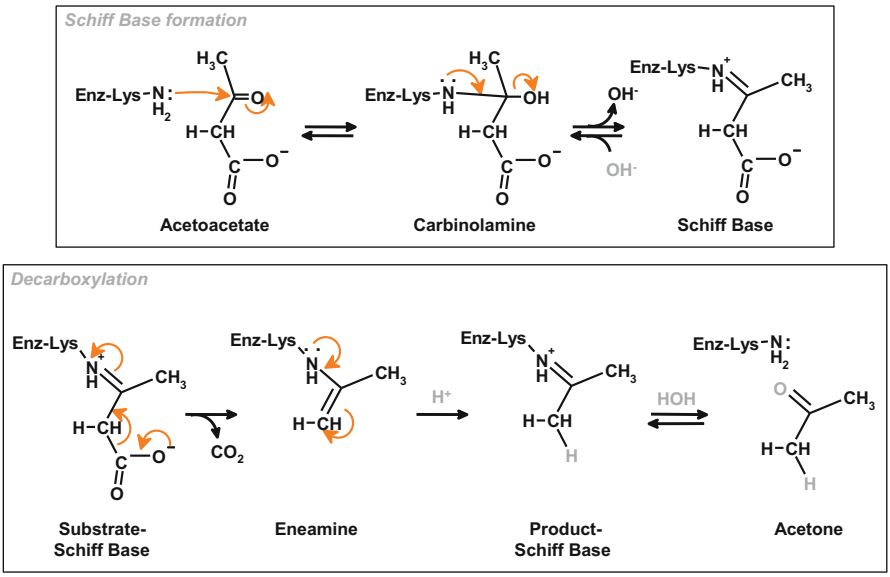
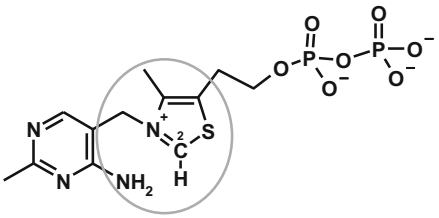


Fig. 34.6 **Acetoacetate decarboxylase reaction mechanism**. Formation of Schiff base between the amino group and a carbonyl is a pH-dependent reversible process. During this process carbonyl oxygen gets equilibrated with the oxygen of water. The reversible protonation of enamine on the enzyme by solvent protons (shown in gray in bottom panel) is responsible for observed deuterium exchange in D_2O. Note that acetone is symmetric and all its methyl hydrogens are equivalent for this exchange

The proposed acetoacetate decarboxylase chemical mechanism is supported by several lines of experimental evidence: (a) sodium borohydride ($NaBH_4$) inactivates this enzyme in the presence of acetoacetate, (b) a lysine residue is radiolabeled by $NaBH_4$ reduction in the presence of 3-[^{14}C]-acetoacetate (or [^{14}C]-acetone), (c) the ε-NH_2 of this active site lysine has an unusually low pKa, (d) the enzyme incorporates ^{18}O from $H_2{}^{18}O$ into the product acetone, and (e) the enzyme catalyzes the exchange of deuterium from D_2O into acetone to produce CD_3COCD_3. In sum, these data justify the *formation of an initial substrate imine intermediate and of an enamine of acetone* in the acetoacetate decarboxylase reaction mechanism. In contrast, an imine intermediate does not form in the case of Mn^{2+}-requiring acetoacetate decarboxylase (Fig. 34.5). Accordingly, $NaBH_4$ inactivation and ^{18}O exchange is also not observed with this Mn^{2+}-dependent catalysis.

34.5 Thiamine Pyrophosphate and α-Keto Acid Decarboxylations

Unlike β-keto acids, α-keto acids are difficult to decarboxylate. The α-carbonyl group of the α-keto acid cannot be used as an electron sink, and it cannot stabilize the developing carbanion. Hence all α-keto acid decarboxylations need cofactor



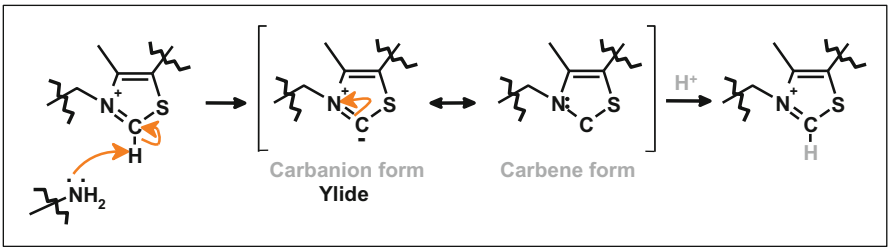


Fig. 34.7 Thiamine pyrophosphate and its thiazolium ring. The C-2 proton is quite acidic and can exchange with the solvent water. Along with the neighboring positive charge on N, TPP carbanion is an ylide

assistance. Nature has found an elegant solution to this chemical problem by recruiting thiamine pyrophosphate (TPP) as an external electron sink. The thiazolium ring of TPP actually participates in the decarboxylation chemistry (Frank et al. 2007; Jordan 2003). The following chemical features make thiazolium (and hence TPP) an ideal cofactor:

1. The C-2 proton of the thiazolium ring is very acidic (dissociates with a $t_{½}$ of 2 min at pH 5.0). This is because (a) the C-2 carbon is sandwiched between two electronegative atoms (N and S) and (b) in its active conformation, proximal amine N on pyrimidine ring helps this deprotonation.
2. The carbanion at C-2 (Fig. 34.7) initiates the nucleophilic attack to the substrate carbonyl. The five-member ring thiazolium (with –N = C-S-) is best suited for the purpose (both on thermodynamic and kinetic grounds), whereas imidazolium (with –N = C-N-) and oxazolium (with –N = C-O-) are not. The carbanion at C-2 with the neighboring positively charged N atom is actually an **ylide** – a neutral dipolar molecule with formal positive and negative charges on adjacent atoms.
3. The thiazolium carbon–nitrogen double bond (and its cationic imine) acts as an electron sink to stabilize the substrate carbanion. This arrangement is quite similar to the cationic imine of Schiff base used in decarboxylating acetoacetate (see Fig. 34.6). Thus TPP furnishes the required electron sink for α-keto acid decarboxylations.

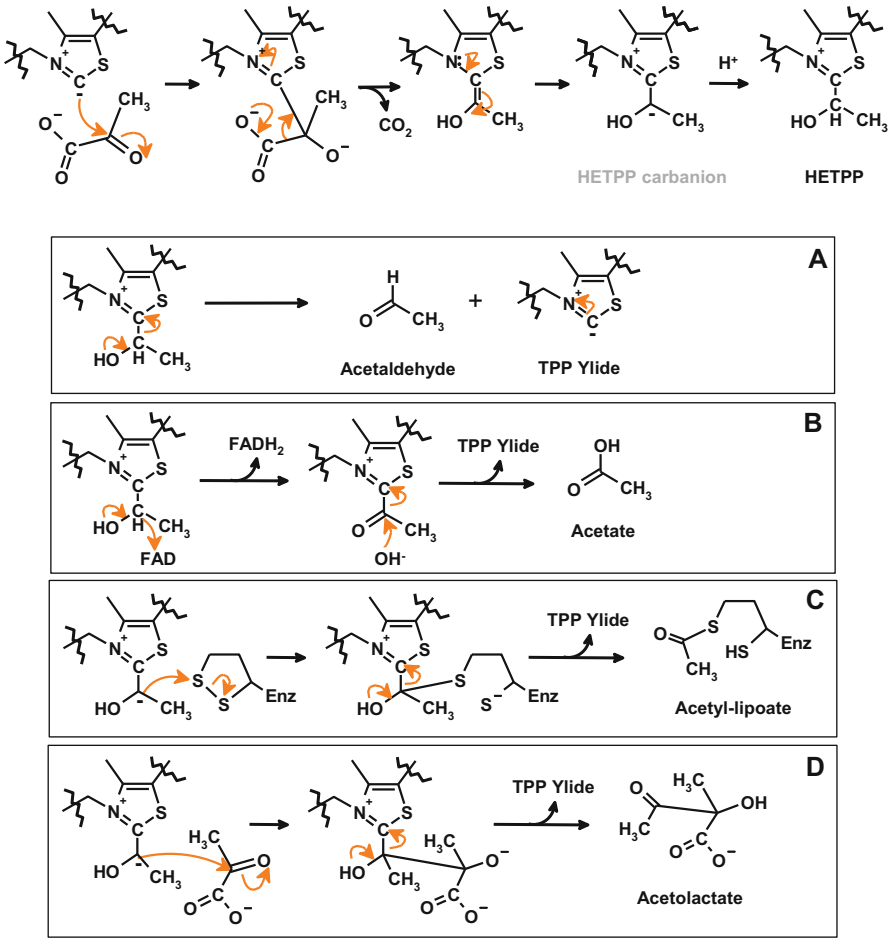


Fig. 34.8 Different decarboxylation reactions of pyruvate involving TPP. Formation of hydroxyethyl TPP (HETPP) carbanion and its subsequent fates are shown. (**a**) Protonation of HETPP and regeneration of coenzyme ylide gives acetaldehyde (non-oxidative decarboxylation; yeast pyruvate decarboxylase). (**b**) Oxidation of HETPP to acetyl-TPP followed by hydrolysis yields acetate (oxidative decarboxylation; *E. coli* pyruvate oxidase). (**c**) When HETPP carbanion reacts with a disulfide (lipoamide), with subsequent coenzyme ylide release, acetyl thioester is formed (oxidative decarboxylation; pyruvate dehydrogenase complex). (**d**) Attack of HETPP carbanion on keto carbon of another pyruvate molecule leads to α-acetolactate (condensation; α-acetolactate synthase)

Decarboxylation of pyruvate is an important example of TPP-assisted electrophilic catalysis. There are many variants of pyruvate decarboxylation. In every case the ylide carbanion (of TPP) first attacks the keto group of pyruvate and forms a lactyl adduct. Enzyme active site electrostatics (Fig. 6.3; Chap. 6) contributes to expulsion of CO_2. Decarboxylation of the adduct results in the carbanion of hydroxyethyl TPP (HETPP; Fig. 34.8). HETPP is a stabilized carbanion since the "**>C = N$^+$<**" moiety of TPP acts as an electron sink – by forming an enamine

intermediate. The fate of HETPP carbanion depends on the type of reaction catalyzed by that particular enzyme. All these possibilities are shown in Fig. 34.8.

Many reaction intermediates in TPP catalysis were recently observed by crystallography. For instance, pyruvate oxidase enzyme forms bound with 2-lactyl TPP (or its stable phosphonate analog), enamine of TPP, and 2-acetyl-TPP provide snapshots of its chemical mechanism.

Oxidative decarboxylation of pyruvate is central to aerobic energy metabolism. This is done by a multi-enzyme complex of three distinct activities: E1, pyruvate decarboxylase; E2, transacetylase; and E3, dihydrolipoyl dehydrogenase.

Partial Reactions of Pyruvate Dehydrogenase Complex

$$\text{Pyruvate} + \text{E1-TPP} \rightarrow \text{E1-HETPP} + \text{CO}_2$$

$$\text{E1-HETPP} + \text{E2-Lipoate} + \text{CoASH} \rightarrow \text{Acetyl CoA} + \text{E1-TPP} + \text{E2-Dihydrolipoate}$$

$$\text{E2-Dihydrolipoate} + \text{NAD}^+ \rightarrow \text{E2-Lipoate} + \text{NADH} + \text{H}^+$$

Overall reaction stoichiometry:

$$\text{Pyruvate} + \text{NAD}^+ + \text{CoASH} \rightarrow \text{Acetyl CoA} + \text{NADH} + \text{CO}_2$$

The acetyl-lipoamide thioester formed by the first enzyme (E1) is used in the next step to generate acetyl-CoA. The third activity (E3, with FAD as the redox device) oxidizes dihydrolipoate back by reducing NAD^+. Mechanism of this dehydrogenase resembles that of glutathione reductase described in the previous chapter (Fig. 33.4; Chap. 33). Metabolic significance of pyruvate dehydrogenase complex is obvious from the range of five cofactors used in its chemistry – TPP, FAD, NAD^+, CoASH, and lipoate. Two other α-keto acid dehydrogenase complexes mechanistically very similar to pyruvate dehydrogenase complex are 2-oxoglutarate dehydrogenase complex of Krebs cycle (2-oxoglutarate → succinyl-CoA) and the branched chain keto acid dehydrogenase complexes of valine, isoleucine, and leucine catabolism.

Electrophilic catalysis and stabilization of substrate carbanion are hallmarks of TPP-dependent decarboxylations. For the same reasons, TPP is an ideal cofactor for carbon–carbon bond formation chemistry as well. The two-carbon transfers are catalyzed by the family of TPP-dependent transketolases. In a transketolase reaction, the TPP carbanion (thiazolium ylide) attacks the carbonyl carbon of a ketose sugar (Fig. 34.9). Instead of a decarboxylation, a carbon–carbon bond cleavage occurs in this adduct, and an aldose (two carbons short!) is released as the first product. In an exact reversal of this reaction sequence, the glycolyl-TPP carbanion now attacks an aldose (same or a different one), and the 2-hydroxyacetyl group is transferred.

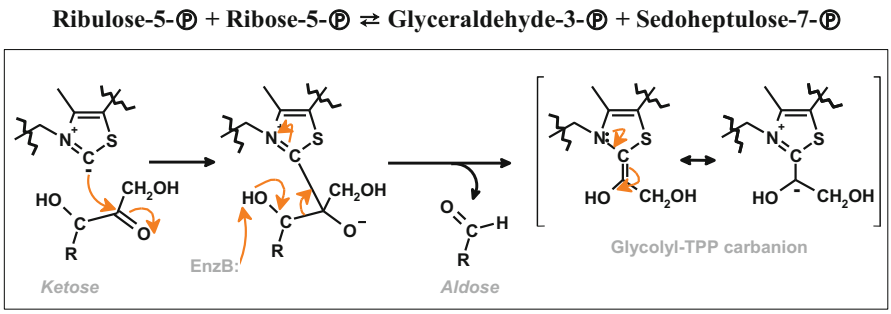


Fig. 34.9 Transketolase mechanism involving a glycolyl-TPP carbanion. Reaction for the synthesis of sedoheptulose-7-Ⓟ is shown, for example. Glyceraldehyde-3-Ⓟ is released from ribulose-5-Ⓟ, while the glycolyl group is retained on the TPP. In a reversal of these steps, ribose-5-Ⓟ accepts the glycolyl group to form sedoheptulose-7-Ⓟ

The 2-hydroxyacetyl (also called the α-ketol) group transfers are critically important reactions of pentose phosphate pathway and the Calvin cycle. These are *two-carbon transfers* brought about by TPP-dependent transketolases. Transaldolases on the other hand carry out *transfer of three-carbon fragments* (dihydroxyacetone equivalents). Between them, transaldolases and transketolases move one-carbon equivalents (CH_2O) around and bring each carbon of every sugar into the metabolic pool. Indeed the two activities together contribute to build glucose (from six individually fixed CO_2 molecules) and also generate the variety of aldoses and ketoses required for cellular metabolism.

34.6 Summing Up

Carbon dioxide is the substrate for carboxylation and product of decarboxylation in a number of biological reactions.

Both CO_2 and HCO_3^- could serve as carboxylation substrates. In carboxylation a suitable carbanion captures either one of these species. But HCO_3^- is not as good an electrophile as CO_2. Prominent cofactors used in these reactions include divalent metal ions, biotin, and vitamin K. Besides, input of energy (in terms of phosphoryl group transfer) may be required to activate HCO_3^-.

Decarboxylations invariably result in the release of CO_2 as the product. Since carbon dioxide is rapidly hydrated to HCO_3^- in water, decarboxylation steps are irreversible and thermodynamically downhill. A carbanion develops when –COOH is lost as carbon dioxide. Stabilizing such a carbanion transition state is an essential trick of enzyme catalysis. The developing negative charge on the C atom could be handled through suitably placed temporary electron sinks. Such sinks may be found on the substrate itself (β-keto group), on the enzyme (Schiff base through Lys-NH_2), or on the cofactor (a divalent metal ion, TPP, or pyridoxal phosphate).

Carboxylation reactions lead to carbon capture (fixing atmospheric CO_2) and decarboxylations release CO_2 (an end product of respiration). The two are complementary in preserving the atmospheric CO_2 balance.

References

Berg IA (2011) Ecological aspects of the distribution of different autotrophic CO_2 fixation pathways. Appl Environ Microbiol 77:1925–1936

Berg IA, Kockelkorn D, Ramos-Vera WH, Say RF, Zarzycki J, Hügler M, Alber BE, Fuchs G (2010) Autotrophic carbon fixation in archaea. Nat Rev Microbiol 8:447–460

Frank RAW, Leeper FJ, Luisi BF (2007) Structure, mechanism and catalytic duality of thiamine-dependent enzymes. Cell Mol Life Sci 64:892–905

Griffiths H (2006) Designs on Rubisco. Nature 441:940–941

Jordan F (2003) Current mechanistic understanding of thiamin-dependent enzymatic reactions. Nat Prod Reports 20:184–201

Knowles JR (1989) The mechanism of biotin-dependent enzymes. Annu Rev Biochem 58:195–222