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MINIREVIEW

Carboxylases in Natural and Synthetic Microbial Pathways▽†

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Carboxylases are among the most important enzymes in the biosphere, because they catalyze a key reaction in the global carbon cycle: the fixation of inorganic carbon (CO_2). This minireview discusses the physiological roles of carboxylases in different microbial pathways that range from autotrophy, carbon assimilation, and anaplerosis to biosynthetic and redox-balancing functions. In addition, the current and possible future uses of carboxylation reactions in synthetic biology are discussed. Such uses include the possible transformation of the greenhouse gas carbon dioxide into value-added compounds and the production of novel antibiotics.

Carboxylases (sensu stricto) are enzymes that catalyze the incorporation of a CO_2 molecule into an organic substrate. Although fixation of CO_2 in an organic substrate is common among all carboxylating enzymes, the underlying mechanisms of the carboxylation reactions differ in essential ways with respect to cosubstrate, cofactor, or metal requirements (see Table S1 in the supplemental material) (8, 42).

The physiological roles of carboxylases are as diverse as their mechanisms. In this review, five different types of carboxylases are functionally defined and discussed. In addition to their well-known functions in (i) CO_2 fixation through the Calvin-Benson-Bassham (CBB) cycle (and other autotrophic pathways), carboxylases also serve an important role in (ii) acquisition of carbon via assimilatory reaction sequences as well as in (iii) acquisition via so-called anaplerotic (from the Greek "ana-" [up] and "plerotikos" [to fill]) reaction sequences, a fact that is generally overlooked. Moreover, carboxylases (iv) supply precursors for important biosynthetic processes such as fatty acid or antibiotic biosynthesis, and their role in (v) redox metabolism was recently unraveled (see Table S1 in the supplemental material).

The first part of this review aims at a comprehensive overview of the different functions of carboxylases and the underlying principle of the corresponding carboxylation reactions. This allows the reader to explore the chemical and functional diversity within this enzyme class and explains why nature has taken advantage of the carboxylating principle in so many different pathways. Such an understanding is essential for the second part of this review, in which the chances and challenges for a use of carboxylases in novel ("synthetic") microbial pathways are discussed.

CARBOXYLASES IN NATURAL MICROBIAL PATHWAYS

Microbial metabolism is very complex, and yet there are only two principal ways in which microorganisms obtain carbon for the synthesis of biomass: autotrophy and heterotrophy. The most basic method is autotrophy, the mechanism by which organisms derive all cell biomass from inorganic carbon (i.e., CO_2). This method requires the fixation of CO_2; consequently, the term "autotrophic carboxylases" refers here to all carboxylating enzymes that serve in these autotrophic pathways and allow the direct transformation of inorganic carbon into central precursor molecules, such as pyruvate, acetyl-coenzyme A (acetyl-CoA), or citric acid cycle (tricarboxylic acid [TCA] cycle) intermediates (Fig. 1). In contrast, heterotrophy—the mechanism by which organisms obtain their cell carbon from organic substrates—does not necessarily require the fixation of CO_2. However, some of the organic growth substrates can be degraded and transformed into biomass only through an essential carboxylation step. Accordingly, carboxylases that function in the dedicated heterotrophic pathways that allow the transformation of organic compounds into central precursor molecules (e.g., TCA cycle intermediates) are referred to here as "assimilatory carboxylases" (Fig. 1). Independently of an organism's method of obtaining carbon, once central metabolites are formed, they are drained for biosynthesis (e.g., amino acid biosynthesis), which ultimately leads to a depletion of TCA cycle intermediates. In order to refill intermediates of the TCA cycle, many organisms (including those that do not rely on the action of autotrophic or assimilatory carboxylases) make use of anaplerotic reactions, which often employ a carboxylation reaction (74, 75). Thus, "anaplerotic carboxylases"—as specified here—are enzymes that mainly serve in TCA cycle-refilling reactions (Fig. 1). Whereas all carboxylases discussed so far ultimately provide central precursors, "biosynthetic carboxylases" form a separate class of carboxylating enzymes that operate in biosynthetic pathways starting from central intermediates (Fig. 1). Finally, the term "redox-balancing carboxylases" is used for enzymes that function mainly in removing excess reducing equivalents [NAD(P)H] during metabolism by using CO_2 as an electron acceptor (Fig. 1). However, it has to be noted that such a systematic classification of carboxylases is arbitrary to some extent, because some of the

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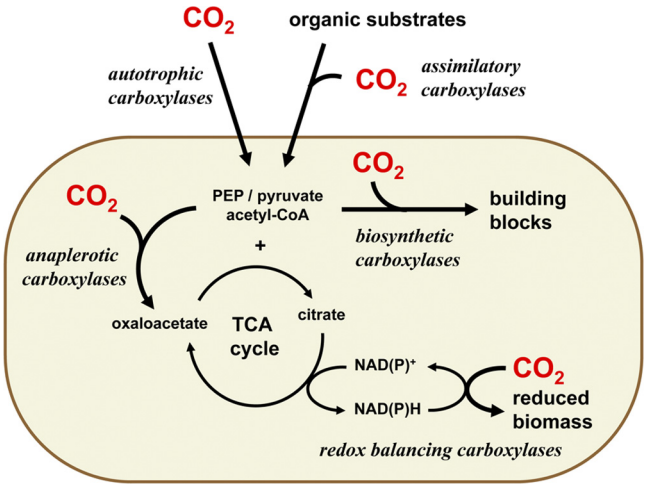

FIG. 1. Physiological classification of carboxylases. The scheme illustrates the five different physiological functions of carboxylases defined in this article. Autotrophic carboxylases serve in autotrophic pathways that allow the formation of central precursor molecules (PEP, pyruvate, acetyl-CoA, and TCA cycle intermediates) solely from inorganic carbon (CO_2). Assimilatory carboxylases function in heterotrophic pathways that convert an organic growth substrate into central precursor molecules. Anaplerotic carboxylases are involved in refilling TCA cycle intermediates when those are drained for biosynthesis. Biosynthetic carboxylases operate in the synthesis of cellular building blocks from central precursor molecules (e.g., α-carboxylic thioesters for fatty acid and polyketide biosynthesis). Redox-balancing carboxylases operate in the transfer of reducing equivalents onto carbon dioxide as terminal electron acceptors and are important for redox homeostasis.

enzymes actually serve more than one physiological function and—at the same time—some physiological functions overlap. As an example, acetyl-CoA/propionyl carboxylase of *Chloroflexus aurantiacus* not only is important for autotrophic CO_2 fixation but also operates in acetyl-CoA assimilation as well as in fatty acid biosynthesis (133, 135). Similarly, crotonyl-CoA carboxylase/reductase, which serves in the ethylmalonyl-CoA pathway for acetyl-CoA assimilation, can be regarded as an assimilatory carboxylase but also fulfils an anaplerotic function (40, 41). Thus, the reader is asked to take the classification of carboxylases presented here as a useful guideline rather than as a fixed rule.

CARBOXYLASES IN AUTOTROPHIC PATHWAYS

In autotrophic pathways that allow the transformation of inorganic carbon into biomass, carboxylases catalyze the most essential reaction: the fixation of carbon dioxide. It is estimated that more than 98% of the CO_2 that enters the biological carbon cycle is fixed by carboxylases, which makes them crucial players in controlling atmospheric CO_2 levels (53, 119). Moreover, since all ecosystems ultimately depend on organic carbon, "autotrophic carboxylases" basically feed all life on earth (67).

About 10^{17} g (100 Gt) of carbon dioxide is transformed into organic compounds per year (53). Most of this process has been traditionally ascribed to the CBB cycle, the autotrophic CO_2 fixation pathway that nourishes plants, algae, and some bacteria (13, 67). Given the global importance of this process, it is not surprising that the quantitatively most abundant enzyme on earth is believed to be ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), the key enzyme of the CBB cycle (35). It has been estimated that about 4×10^{13} g (40 Mt) of RubisCO exists in the biosphere, which would correspond to an intangible 5 kg of RubisCO per person on earth (94).

In addition to the CBB cycle, there are five other known autotrophic CO_2 fixation pathways (see Fig. S1 to S4 in the supplemental material): (i) the reductive citric acid cycle (47), (ii) the reductive acetyl-CoA pathway (100), (iii) the 3-hydroxypropionate (or Fuchs-Holo) bicycle (61, 134), (iv) the hydroxypropionate/hydroxybutytrate cycle (16), and (v) the dicarboxylate/hydroxybutyrate cycle (64). Like the CBB cycle, these "alternative" autotrophic pathways all use carboxylases as key enzymes for the fixation of CO_2. The only exception is the reductive acetyl-CoA pathway (see Fig. S1 in the supplemental material), in which two molecules of CO_2 are initially converted into acetyl-CoA by reduction via formate dehydrogenase and CO dehydrogenase/acetyl-CoA synthase (98). However, the reductive acetyl-CoA pathway may contribute up to only 2% of the global carbon fixation (119). Moreover, transformation of the initial acetyl-CoA fixation product into more complex molecular compounds through this pathway in fact requires a carboxylation reaction (via pyruvate:ferredoxin oxidoreductase; see below) such that, in organisms using the reductive acetyl-CoA pathway, approximately five-sixths of the carbon is fixed via CO_2 reduction and one-sixth is fixed via carboxylation (54, 98, 100).

Each of the six known autotrophic pathways appears to be tightly connected to the physiology of a given organism in its ecological niche (15, 17, 18, 118). Hence, none of the natural CO_2 fixation pathways represents the optimal solution; rather, each is the result of an optimized evolutionary adaption. The preference of a given organism for one of these six pathways strongly depends on important physiological factors, such as energy requirements (with respect to the number of moles of ATP per mole of CO_2 fixed) or the nature of reducing equivalents for the subsequent reduction of the CO_2 group fixed [NAD(P)H, ferredoxin]. Other determinants are the oxygen (in)sensitivity of the pathway's reactions and their demand for special cofactors or metals (17, 18). As discussed below, many of these parameters are directly connected to the central carboxylation reaction, which makes autotrophic carboxylases crucial elements in the physiology of a CO_2 fixation pathway.

rTCA cycle (see Fig. S2 in the supplemental material). The reductive citric acid cycle is generally considered the most energy-efficient CO_2 fixation pathway (~0.6 mol ATP/mol CO_2 for pyruvate) (15, 17, 23, 47). This is reflected by the activity of the pathway's key carboxylases, isocitrate dehydrogenase, α-ketoglutarate:ferredoxin oxidoreductase, and pyruvate:ferredoxin oxidoreductase. All three enzymes couple their carboxylation reaction to a subsequent reduction step. Whereas isocitrate dehydrogenase is an NAD(P)H-dependent enzyme, the latter two enzymes use ferredoxin as a reductant (Fig. 2) (2, 82, 99, 107). Such reductive carboxylations are catalytically highly efficient (see below) and contribute essentially to the low energy requirement of the reductive TCA (rTCA) cycle. This is particularly striking in comparison to the energy demands of other autotrophic CO_2 fixation pathways (e.g., the CBB cycle; see below) that employ nonreducing carboxylases.

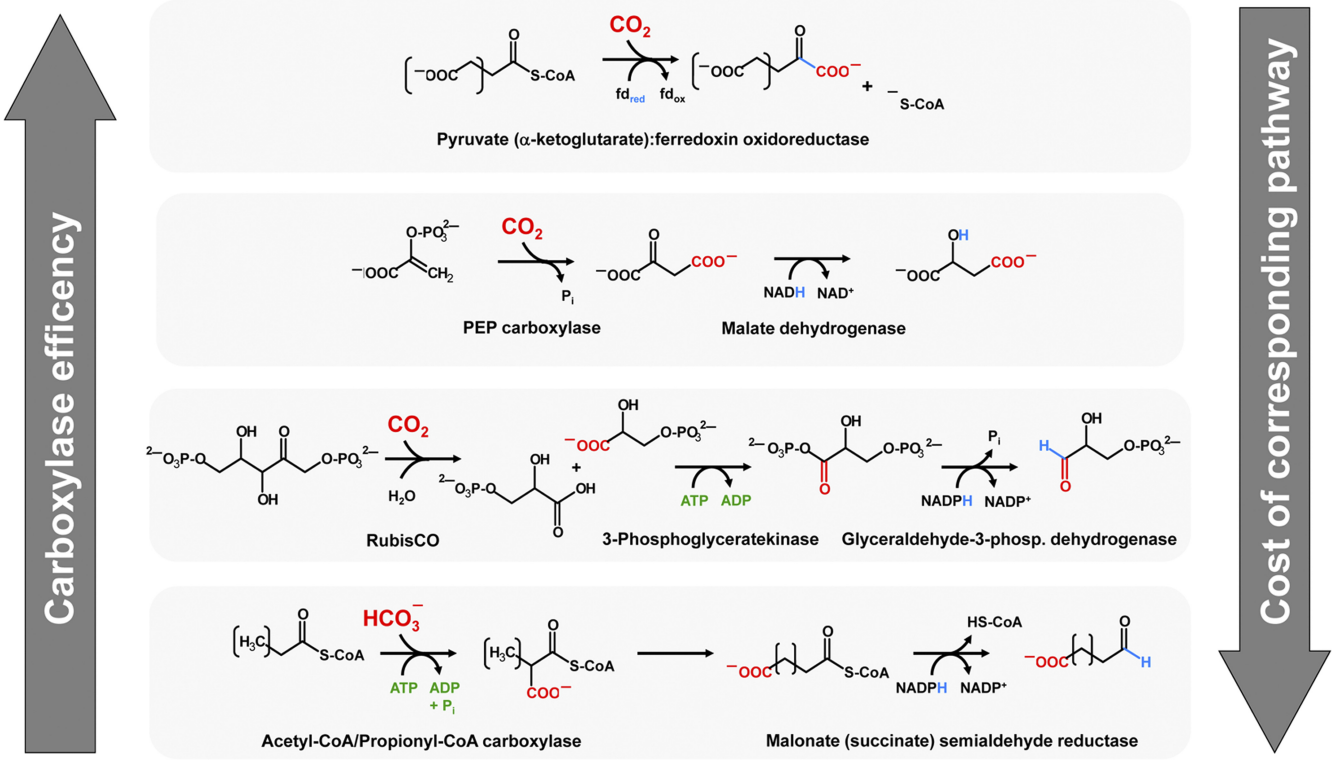

FIG. 2. Autotrophic carboxylation reactions. All carboxylases used in autotrophic CO_2 fixation pathways are listed and sorted according to "efficiency." Carboxylases that do not require ATP hydrolysis for their carboxylation reaction are considered to be more efficient ("energetic efficiency"). Similarly, carboxylases that combine carboxylation with reduction, or allow a subsequent reduction step without extra ATP hydrolysis, are also considered to be more efficient ("catalytic power"). Note that carboxylase efficiency is directly linked to the total energetic costs of a given pathway (see the text for a detailed discussion).

However, reducing carboxylases of the ferredoxin type are highly oxygen sensitive because they contain iron-sulfur clusters that are easily oxidized (2, 72). The oxygen sensitivity of these enzymes limits ferredoxin-dependent carboxylations—and therefore also the rTCA cycle—to certain anoxic environments. Accordingly, the rTCA cycle is mainly found to occur in anaerobic and microaerobic bacteria, such as green sulfur bacteria, and in many delta- and epsilonproteobacteria, as well as in members of the *Aquificae* clade (14, 65, 108). Interestingly, the thermophile *Hydrogenobacter thermophilus* (order *Aquificales*) is known to use the rTCA cycle under aerobic conditions as well. *H. thermophilus* encodes two different isoforms of α-ketoglutarate:ferredoxin oxidoreductase (127, 128, 132). One isoform is the canonical oxygen-sensitive carboxylase (composed of two subunits) that is preferentially expressed under anoxic conditions (128). The other isoform is an α-ketoglutarate:ferredoxin oxidoreductase complex of five subunits, which is more oxygen tolerant and synthesized under oxic conditions (127, 128, 132). However, the catalytic efficiency of the oxygen-tolerant enzyme is reduced by an order of magnitude compared to that of oxygen-sensitive α-ketoglutarate:ferredoxin oxidoreductases (127, 129). This apparent tradeoff between oxygen sensitivity and carboxylase activity might provide an explanation of why the rTCA cycle is mainly restricted to anaerobic and microaerobic organisms.

CBB cycle (see Fig. S3 in the supplemental material). Compared to the rTCA cycle, the CBB cycle is a more energy-expensive CO_2 fixation strategy (~2.3 mol ATP/mol CO_2 for pyruvate) (13, 15, 17, 23). The difference in energetic cost can be largely attributed to the pathway's carboxylase RubisCO, which catalyzes the carboxylation of ribulose-1,5-bisphosphate into two molecules of 3-phosphoglycerate. RubisCO's nonreductive carboxylation mechanism requires the reduction of the initial fixation product by a separate enzyme, glyceraldehyde-3-phosphate dehydrogenase (Fig. 2). Compared to the reductive carboxylations of the rTCA cycle, this separation of carboxylation and reduction in the CBB cycle requires extra energy, because the CO_2 group that is fixed has to be activated by phosphorylation for the subsequent reduction. The extra ATP hydrolyzed in this reaction sequence accounts for almost 60% of the total energetic difference between the CBB cycle and rTCA cycle, which demonstrates the dramatic influence of a carboxylase on the total energy balance and thus the physiology of an autotrophic pathway. Importantly, RubisCO and glyceraldehyde-3-phosphate dehydrogenase are both oxygen insensitive, which is believed to be the major reason for the wide distribution of the CBB cycle in strictly aerobic and facultatively anaerobic autotrophic organisms (15).

However, even though RubisCO itself is oxygen insensitive, it exhibits a wasteful oxygenase side-reaction in which ribulose-1,5-bisphosphate is oxidized by molecular oxygen into one molecule of 3-phosphoglycerate and one molecule of 2-phosphoglycolate. RubisCOs that operate under highly aerobic conditions (e.g., cyanobacterial RubisCOs) were previously

shown to discriminate better between the two alternative substrates CO_2 and O_2 (10). Interestingly, these "aerobic" RubisCOs are in general also less catalytically efficient than their more unspecific counterparts found in facultatively anaerobic organisms (e.g., purple nonsulfur bacteria). This points to a tradeoff between oxygen "sensitivity" and enzyme activity in RubisCO (10, 11) that resembles the situation seen with the different α-ketoglutarate ferredoxin oxidoreductase isoforms. Yet it is believed that all RubisCOs are nearly perfectly optimized to the environmental conditions of the host organism, balancing enzyme activity with oxygen specificity as minimally as possible or necessary (10, 11, 115). In fact, many facultatively anaerobic proteobacteria carry multiple RubisCO isoforms that are differentially expressed under changing conditions, most notably various ratios of CO_2 to O_2 (131).

Fuchs-Holo bicycle (see Fig. S4 in the supplemental material). The Fuchs-Holo or 3-hydroxypropionate (3HP) bicycle is considered to be as energetically expensive as the CBB cycle (~2.3 mol ATP/mol CO_2 for pyruvate) (15, 17, 23, 61, 134). As in the case of the CBB cycle, the energy difference between the 3HP bicycle and the rTCA cycle can be largely ascribed to nature of the pathway's central carboxylase. In the 3HP bicycle, CO_2 is fixed by a putatively bifunctional acetyl-CoA/propionyl-CoA carboxylase that yields malonyl-CoA (from acetyl-CoA) and methylmalonyl-CoA (from propionyl-CoA) (66, 114). In contrast to the reductive carboxylases of the rTCA (or CBB's RubisCO), carboxylation by acetyl-CoA/propionyl-CoA carboxylase requires ATP. Acetyl-CoA/propionyl-CoA carboxylase is a biotin-dependent enzyme that uses the biotin cofactor to deliver CO_2 to the active site in form of a covalent CO_2-biotin adduct, the so-called carboxybiotin (56). Formation of carboxybiotin requires an activated CO_2 molecule (carboxyphosphate) that is ultimately generated from HCO_3^- by ATP hydrolysis (73, 88). However, although carboxylation through the activity of acetyl-CoA/propionyl-CoA carboxylase is energy expensive, the subsequent reduction reactions of the carboxylation products do not require additional energy input (Fig. 2). Taking these findings together, this makes initial CO_2 fixation via the 3HP bicycle and that via the CBB cycle energetically *de facto* equivalent. Compared to the rTCA cycle, each of the two pathways causes an extra cost of one molecule of ATP that is directly linked to the central carboxylation reaction. In the case of the 3HP bicycle, this extra ATP comes from the biotin-dependent carboxylation reaction itself, whereas for the CBB cycle, the extra ATP molecule is required for the subsequent activation of the carboxylation product for reduction. For CBB's RubisCO, however, the extra costs for CO_2 fixation via the 3HP bicycle come with the advantage that carboxylation by acetyl-CoA/propionyl-CoA carboxylase is oxygen insensitive. Consequently, the 3HP bicycle functions in the facultatively aerobic green nonsulfur bacterium *Chloroflexus aurantiacus* (61).

HP/HB cycle (see Fig. S5 in the supplemental material) and DC/HB cycle (see Fig. S6 in the supplemental material). Finally, the recently discovered hydroxypropionate/hydroxybutyrate (HP/HB) cycle and a closely related variant, the dicarboxylate/hydroxybutyrate (DC/HB) cycle, may represent the best examples of the manner in which the physiology of autotrophic CO_2 fixation is determined by the central carboxylating enzymes (16, 64). Both CO_2 fixation pathways share many common steps and intermediates, especially those involved in the regeneration of the initial CO_2 acceptor molecule, acetyl-CoA. Yet the two pathways differ essentially in their CO_2-fixing reactions. Whereas the HP/HB cycle uses biotin-dependent acetyl-CoA/propionyl-CoA carboxylase (16, 66), the DC/HB cycle employs pyruvate:ferredoxin oxidoreductase and phosphoenolpyruvate (PEP) carboxylase (64) (Fig. 2). This difference in the CO_2-fixing reactions of the two pathways is perfectly reflected by a difference in energy requirement and oxygen sensitivity. Reductive carboxylation as used in the DC/HB cycle corresponds to an energetically more favorable pathway (~1.6 mol ATP/mol CO_2 for pyruvate) compared to that seen with the HP/HB cycle (~3 mol ATP/mol CO_2 for pyruvate), which uses energy-expensive, biotin-dependent carboxylation reactions (15, 17, 23). On the other hand—and in line with the oxygen sensitivity of its ferredoxin-dependent carboxylase—the DC/HB cycle seems to be limited to anaerobic autotrophic species of the *Thermoproteales* and *Desulfurococcales* order (18, 64, 102). In contrast, the HP/HB cycle was found to operate in (micro)aerobic members of the *Sulfolobales* order, as well as in aerobic marine group I archaea ("Taumarchaeota") (16, 18, 19).

In summary, these examples demonstrate that carboxylases are key enzymes in autotrophic CO_2 fixation that have an important influence on the physiology and distribution of a given CO_2 fixation pathway. The "efficiency" of a carboxylase generally corresponds well to the total energy requirements of the whole CO_2 fixation pathway (Fig. 2); note that enzyme efficiency is defined here as representing a combination of energetic efficiency (i.e., carboxylation is ATP independent) and catalytic efficiency (i.e., the combination of carboxylation and reduction). Starting from pathways that solely use (rTCA) or mainly use (DC/HB cycle) reductive carboxylases and proceeding to pathways that employ nonreducing, biotin-independent carboxylases (CBB cycle) or biotin-dependent carboxylases (3HP bicycle and HP/HB cycle), the amount of ATP required per mole of CO_2 fixed into pyruvate increases from 0.6 mol (rTCA) to 1.6 mol (DC/HB cycle), 2.3 mol (CBB and 3HP bicycle), and, finally, 3 mol (HP/HB cycle). On the other hand, the more efficient the carboxylation reaction, the more oxygen sensitive the corresponding CO_2 fixation pathway seems to be. The rTCA cycle and the DC/HP cycle that both use ferredoxin-dependent reductive carboxylases are found almost exclusively in strictly anaerobic organisms. In contrast, the CBB cycle, the 3HP bicycle, and the HP/HB cycle, which employ "expensive" nonreducing carboxylases, are dominant in facultatively anaerobic and strictly aerobic organisms. Finally, these correlations of carboxylase properties and pathway physiology are of importance for any efforts to improve natural or to design synthetic CO_2 fixation pathways, as discussed below.

CARBOXYLASES IN ASSIMILATORY PATHWAYS

Apart from their role in autotrophic CO_2 fixation, many carboxylases also serve in the microbial assimilation of various organic substrates. In these metabolic pathways, "assimilatory carboxylases" are essential for the introduction of functional groups into the substrates that allow the further transfor-

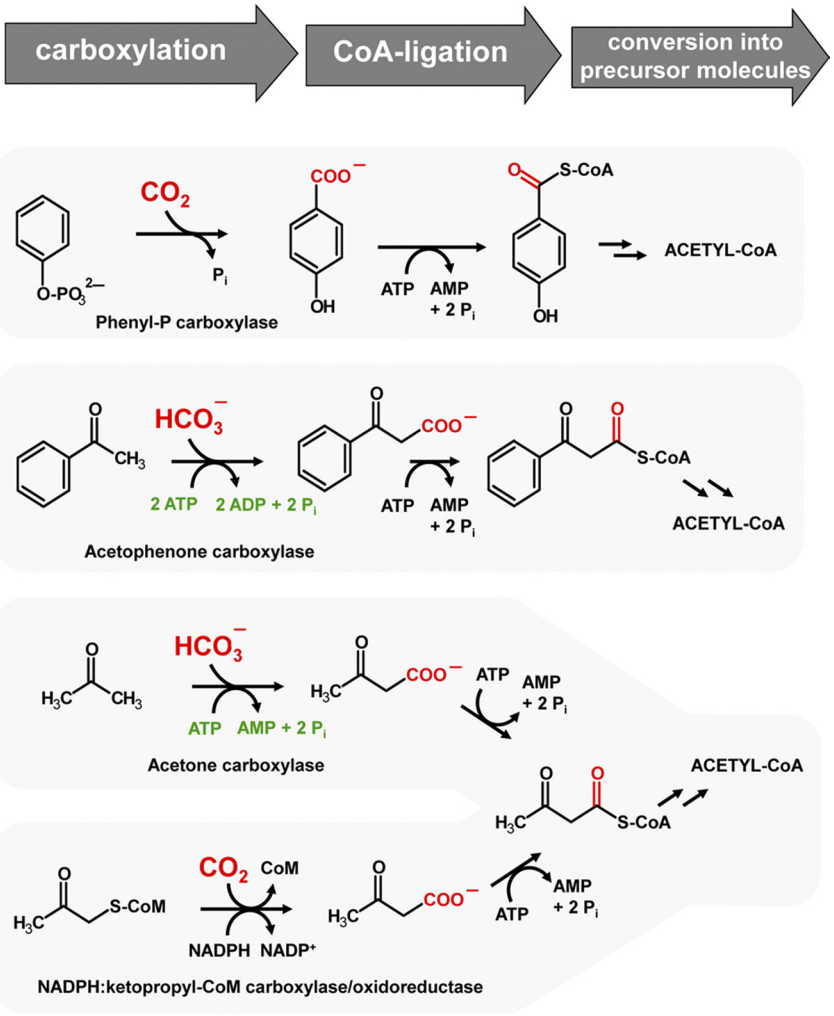

FIG. 3. Assimilatory carboxylation reactions I: substrate functionalization of inert substrates by carboxylation. Many compounds that bear no functional terminal group are assimilated through an initial carboxylation step. The carboxylic acid formed is then transformed into a CoA ester and further channeled into central metabolites (e.g., by classical β-oxidation). Functionalization of substrates by carboxylation is a common strategy under anaerobic conditions, when no molecular oxygen is present to activate the substrate.

mation of these organic compounds into central precursor molecules. Many ecologically relevant compounds are in fact metabolized via "assimilatory carboxylases." Thus, these enzymes are as important for the global carbon cycle as "autotrophic carboxylases," a fact that is generally underappreciated.

Functionalization of inert substrates. The introduction of a carboxylic group is a common strategy in the assimilation of substrates that lack any functionalizable terminal carbon group, such as phenol, acetophenone, epoxypropane, or acetone (39, 55, 70, 106) (Fig. 3). Such carboxylations are an important alternative to oxygen-dependent functionalization reactions and are widely used under anoxic conditions. In anaerobic phenol assimilation of *Thauera aromatica*, phenylphosphate is carboxylated into 4-hydroxybenzoate by phenylphosphate carboxylase (106). Anaerobic metabolism of acetophenone in *Aromatoleum aromaticum* requires acetophenone carboxylase, which yields benzoylacetate (70). Epoxypropane and acetone are transformed into acetoacetate by NADPH:2-ketopropyl-CoM carboxylase/oxidoreductase (*Xanthobacter autotrophicus* Py2) and acetone carboxylase (*Rhodobacter capsulatus*), respectively (38, 39). Subsequently, all these carboxylic acids are activated to their corresponding CoA-esters and further converted to central metabolites.

It is interesting that the method of substrate functionalization by carboxylation has apparently independently evolved multiple times. The most striking example of such convergent evolution is acetoacetate formation in epoxypropane and acetone assimilation (38). Although both pathways yield the same central intermediate (acetoacetate) upon carboxylation, the corresponding carboxylases (NADPH:2-ketopropyl-CoM carboxylase/oxidoreductase and acetone carboxylase) differ structurally and mechanistically (Fig. 3) (39). On the other hand, some of the assimilatory carboxylases share an evolutionary origin. This seems to be the case for acetophenone and acetone carboxylase, which show high sequence similarity and have similar mechanistic features (21, 22, 70, 109). Recently, genes with homology to those of phenylphosphate carboxylase have been identified as important for the anaerobic metabolism of

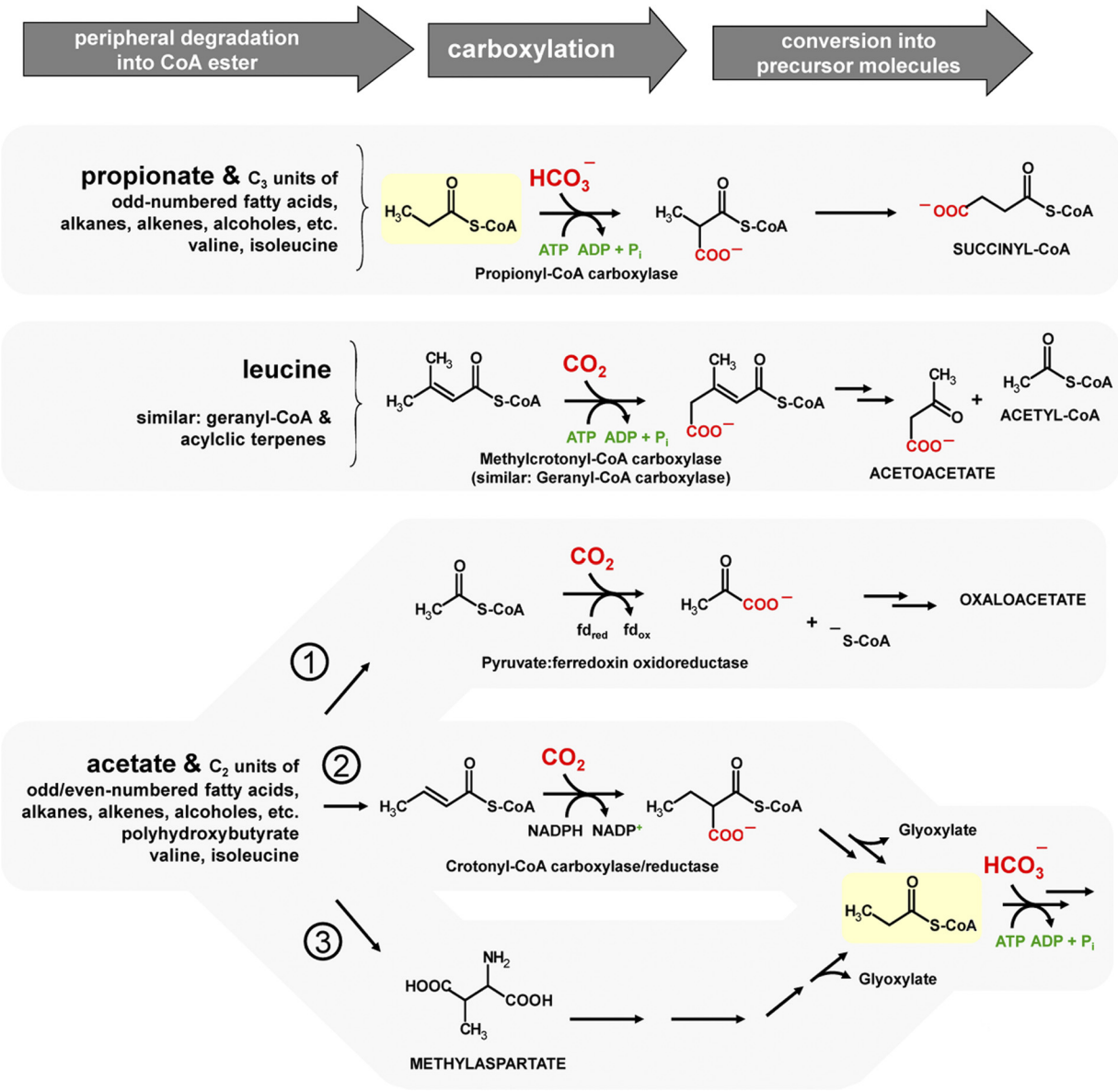

FIG. 4. Assimilatory carboxylation reactions II: propionate, leucine and acetate assimilation. Many compounds are initially channeled into few CoA esters (e.g., propionyl-CoA, acetyl-CoA) during assimilation. The further conversion of these CoA esters into central precursor molecules requires carboxylation. Acetate is transformed initially into acetyl-CoA and can be assimilated via three different carboxylative routes (pyruvate: ferredoxin oxidoreductase [circled "1"]; ethylmalonyl-CoA pathway [circled "2"]; the methylaspartate cycle [circled "3"]. Note the importance of propionyl-CoA carboxylation in many of the assimilation pathways (highlighted in yellow).

benzene in the iron-reducing culture *BF* (1) as well as for naphthalene in the sulfate-reducing enrichment culture *N47* (20). However, whether these genes encode true benzene or naphthalene carboxylases remains to be demonstrated. Finally, the degradation of medium- to long-chain alkanes in the strictly anaerobic Hxd3 sulfate-reducer strain was also suggested to follow an initial carboxylation reaction (25, 111).

Overcoming incomplete β-oxidation: propionate and leucine assimilation. In the assimilation of substrates that already carry functional groups, assimilatory carboxylases are used to introduce a carboxyl group that is important for the further conversion of these molecules into central C_4-building blocks (e.g., succinyl-CoaA, oxaloacetate). This is the case for the assimilation of such common substrates as propionate and leucine and many other compounds that are all initially degraded to form one of these metabolites (Fig. 4). The best-studied example is propionate, which is assimilated in many organisms (including eukaryotes) via propionyl-CoA (89). Because propionyl-CoA is the shortest odd-numbered acyl-CoA ester possible, it cannot be further metabolized via the classical β-oxidation pathway of fatty acids. This biochemical problem is overcome by biotin-dependent propionyl-CoA carboxylase, which yields (2*S*)-methylmalonyl-CoA (89). (2*S*)-Methylmalonyl-CoA is subsequently transformed into the TCA cycle intermediate succinyl-CoA (24). In addition to propionate, the

final C_3 units of odd-numbered fatty acids, alkanes, alkenes, alcohols, and waxes, as well as the branched amino acids valine and isoleucine, are all assimilated via this pathway. Moreover, propionyl-CoA carboxylation is also important in some acetate assimilation pathways (see below).

Similarly to propionate metabolism, assimilation of leucine also requires carboxylation to overcome an incomplete β-oxidation pathway (Fig. 4) (126). In the case of leucine, β-oxidation stops at the level of 3-methylcrotonyl-CoA because the C3 position of the molecule is blocked from further transformation by the branching methyl group. Carboxylation of 3-methylcrotonyl-CoA by biotin-dependent methylcrotonyl-CoA carboxylase yields 3-methylglutaconyl-CoA (50, 51), which is then transformed into three molecules of acetyl-CoA (4, 63). However, in contrast to other acyl-CoA esters, which are usually carboxylated at the α position, the CO_2 group is in this case introduced at the γ position (50, 52, 59). Recently, the genes encoding geranoyl-CoA carboxylase, a homolog of 3-methylcrotonyl-CoA carboxylase, were identified and functionally assigned (4, 63). Geranoyl-CoA carboxylase is involved in the degradation of branched isoprenoids (acylic terpenes) and also employs γ-carboxylation to overcome incomplete β-oxidation (50, 51).

Acetate assimilation (see Fig. S7 in the supplemental material). Finally, acetate and the even-numbered chains of fatty acids, as well as alkanes, alkenes, alcohols, esters, waxes, and the bacterial storage polymer polyhydroxybutyrate (PHA), are all assimilated via acetyl-CoA. In strict anaerobes, acetyl-CoA is generally transformed into pyruvate by pyruvate:ferredoxin oxidoreductase, the same enzyme that functions in the rTCA cycle, the DC/HB cycle, and the reductive acetyl-CoA pathway (117). However, due to the oxygen sensitivity of this enzyme, acetate assimilation via this route is not possible under aerobic conditions; thus, alternative pathways have to exist in (micro)aerobic organisms. For more than 50 years, the glyoxylate shunt, a modified TCA cycle that involves no carboxylation reaction, was the only known aerobic acetyl-CoA assimilation strategy (75). It has been realized only lately that at least two alternative aerobic pathways for acetyl-CoA assimilation exist that make use of a central carboxylation step to convert this C_2 compound into molecular building blocks of greater complexity (C_3 and C_4 intermediates) (Fig. 3; see also fig S7 in the supplemental material) (37, 41, 71, 105).

Recently, the ethylmalonyl-CoA pathway, which operates in ecologically relevant species, such as many alphaproteobacteria (*Methylobacterium*, *Rhodobacter*) and actinomycetes (*Streptomyces*), was discovered (41, 44, 79, 92, 110). In this acetyl-CoA assimilation strategy, two carboxylation reactions occur. First, the C_4 compound crotonyl-CoA (a condensation product of two acetyl-CoA molecules) is carboxylated into the C_5 intermediate (2*S*)-ethylmalonyl-CoA. This reaction is catalyzed by crotonyl-CoA carboxylase/reductase (CCR), which represents a novel class of biotin-independent reductive acyl-CoA carboxylases that use NADPH as a reductant (42). In the ensuing process, ethylmalonyl-CoA is cleaved into the C_3 unit propionyl-CoA and a C_2 intermediate (glyoxylate), which are subsequently transformed into two C_4 building blocks (succinyl-CoA and malate, respectively) (43–45, 136). Transformation of propionyl-CoA into succinyl-CoA involves biotin-dependent propionyl-CoA carboxylase, which catalyzes the second essential carboxylation reaction of the ethylmalonyl-CoA pathway. The other aerobic acetyl-CoA assimilation pathway that makes use of a carboxylation reaction is the methylaspartate cycle discovered very recently in the haloarchaeum *Haloarcula marismortui* (71). In this pathway, the C_5 key intermediate methylaspartate is formed and then—in similarity to the ethylmalonyl-CoA pathway—transformed into glyoxylate and propionyl-CoA. Propionyl-CoA is converted into succinyl-CoA through the same reaction sequence as occurs in the ethylmalonyl-CoA pathway involving propionyl-CoA carboxylase (71), which adds to the importance of this carboxylase in carbon assimilation.

Assimilatory carbon by-fixation. Assimilatory carboxylases not only provide a functional group to the substrate but also allow additional uptake ("by-fixation") of inorganic carbon. Therefore, one could consider carboxylation-dependent assimilatory reaction sequences also to be "partial CO_2 fixation pathways." The amount of carbon dioxide fixed through these pathways can range from the amount seen under conditions of a pure catalytic role to a substantial fraction of CO_2 fixed per substrate molecule assimilated. As an example, during assimilation of phenol and acetate assimilation via the methylaspartate cycle, the CO_2 fixed is only of catalytic nature, because both assimilatory carboxylations are either preceded (methylaspartate cycle) or followed (phenol assimilation) by a decarboxylation reaction. In contrast, assimilation of leucine (leucine + CO_2 → 3 acetyl-CoA) and propionate (propionate + CO_2 → succinyl-CoA) allows the by-fixation of 17% and 25% inorganic carbon, respectively. Finally, carbon assimilation via the ethylmalonyl-CoA pathway (3 acetate + 2 CO_2 → succinyl-CoA + malate) yields a maximum of 33% additional carbon fixed. However, many of these numbers are of only a theoretical nature so far, and the by-fixation of inorganic carbon is expected to strongly depend on the redox status of the carbon substrate assimilated. Yet such carbon by-fixation is in principle possible, in similarity to the carboxylation-dependent redox-balancing mechanisms that are discussed below (85). In fact, the first experimental results, as well as theoretical calculations, indicate the importance of carbon by-fixation in the ethylmalonyl-CoA pathway (76, 93).

Methanol assimilation. In conclusion, carboxylases involved in the assimilation of reduced C_1 compounds such as methanol, methylsulfide, and methylamine should also be mentioned briefly. The phenomenon called "methylotrophy" is actually a very special case of heterotrophy, and microorganisms have established very different solutions, some of which also require carboxylation reactions, for the specific problems raised by methylotrophy (5). The most interesting case is that of the ribulose bisphosphate pathway, as used by bacteria like *Paracoccus denitrificans*, in which methanol is first oxidized completely to carbon dioxide before CO_2 is then refixed via the reactions of the CBB cycle (6, 31). Similarly to the ribulose bisphosphate pathway, the serine cycle that operates in many methylotrophs, such as *Methylobacterium extorquens*, also makes use of (at least) one essential carboxylation reaction (7, 77). However, the reader is referred to current literature for more details on the fascinating physiology of methylotrophy (29, 30, 41, 92, 121).

CARBOXYLASES IN ANAPLEROSIS

In central carbon metabolism, many intermediates are drained from the TCA cycle for biosynthetic purposes. In fact, almost half of all amino acids are directly synthesized from TCA cycle metabolites (54). In order to maintain a functioning TCA cycle, these metabolites are constantly replenished via anaplerotic reactions (see above) (74). The most important anaplerotic reaction in nature is the conversion of pyruvate or phosphoenolpyruvate (PEP) into oxaloacetate (90). This transformation involves an essential carboxylation reaction that can be catalyzed by two different enzymes: phosphoenolopyruvate carboxylase and biotin-dependent pyruvate carboxylase (54, 68, 69). The biological importance of anaplerotic carboxylases is emphasized by the fact that the latter enzyme is highly conserved in many organisms. In this context, it should be remembered that acetyl-CoA assimilation via the ethylmalonyl-CoA pathway and the methylaspartate cycle also functions in anaplerosis, indicating a functional overlap between anaplerotic and assimilatory carboxylases in some organisms (see above).

CARBOXYLASES IN BIOSYNTHETIC PATHWAYS

As described above, "autotrophic carboxylases" and "assimilatory carboxylases" are essential enzymes in central carbon metabolism that are important for the supply of all cell carbon. In contrast, "biosynthetic carboxylases" refers to enzymes that are dedicated to the synthesis of one specific building block in the biosynthesis of one particular class of molecules. Prime examples of biosynthetic carboxylations are the synthetic pathways to fatty acids and polyketide secondary metabolites that involve essential carboxylation reactions. In both biosynthetic pathways, α-carboxylations are used to provide activated acyl-CoA ester building blocks for subsequent carbon-carbon bond-forming reactions (see Fig. S8 in the supplemental material) (32).

In fatty acid biosynthesis of eukaryotes and bacteria, malonyl-CoA extender units for the growing fatty acid chain are generated by carboxylation of acetyl-CoA through biotin-dependent acetyl-CoA carboxylase (104, 120). The incorporated CO_2 is used to activate the acetyl-CoA molecule for the subsequent elongation reaction; irreversible elimination of this CO_2 group by decarboxylation yields a reactive enolate anion that serves as a nucleophile to extend the growing carbon chain in a Claisen condensation-like manner (see Fig. S8 in the supplemental material).

As seen with fatty acid synthases, the phylogenetically related polyketide synthases use α-carboxyl-thioesters as extender units (32). In order to vary the molecule backbone, however, polyketide synthases make use of other extender units in addition to malonyl-CoA. As for malonyl-CoA, most of these extender units are also provided by carboxylation (although some very rare extender units are synthesized independently of carboxylation) (27, 28). Prominent examples are methylmalonyl-CoA, which is supplied by propionyl-CoA carboxylase, and ethylmalonyl-CoA, which is provided by the recently discovered CCR (28, 41). A homolog of the latter enzyme was also shown to be responsible for the synthesis of the unusual extender units chloroethylmalonyl-CoA (from chlorocrotonyl-CoA) and propylmalonyl-CoA (from 2-pentenyl-CoA) (46, 80). Moreover, in the biosynthesis of the proteasome inhibitor cinnabaramide A and the macrolide antibiotic filipin, CCR homologs supply hexylmalonyl-CoA via reductive carboxylation of 2-octenoyl-CoA (97, 112, 130).

In addition to these well-known carboxylation reactions, which operate in fatty acid and polyketide biosynthesis, other biosynthetic carboxylation reactions have been reported more recently. In the methanogenic archaea *Methanobacterium autotrophicum* and *Methanococcus maripaludis*, novel ferredoxin-dependent carboxylases were identified that are linked to biosynthetic pathways. Apart from the well-studied pyruvate:ferredoxin oxidoreductase and α-ketoglutarate:ferredoxin oxidoreductase, those organisms also employ α-keto-isovalerate:ferredoxin oxidoreductase and indolepyruvate:ferredoxin oxidoreductase homologs for the biosynthesis of branched amino acids (e.g., valine) and aromatic amino acids (e.g., tryptophan and phenylalanine) (96, 116).

CARBOXYLASES IN REDOX BALANCING

Carboxylation-dependent redox balancing in purple nonsulfur bacteria (PNSB) such as *Rhodobacter capsulatus*, *Rhodopseudomonas palustris*, and *Rhodobacter sphaeroides* has been studied in detail (48, 49, 76, 84, 85, 124). When these organisms are grown anaerobically in the light on butyrate, a substrate (oxidation state of carbon, −1) that is more reduced than the average cell carbon state seen in bacteria (oxidation state of carbon, between −0.2 and −0.5 [33, 85, 122]), external CO_2 has to be added to the medium. Under such photoheterotrophic conditions, CO_2 fixation via RubisCO is absolutely essential, as demonstrated by genetic knockout studies. Thus, CO_2 might be considered here to be an electron sink that is important to balance excess reducing equivalents from the more reduced growth substrate. The requirement for an operating CBB cycle under these conditions should not be confused with autotrophic CO_2 fixation. In contrast to autotrophy, CO_2 fixation in photoheterotrophic carbon assimilation of PNSB is in fact not really necessary for carbon supply but is necessary exclusively for redox balancing. This is shown by the fact that the aforementioned RubisCO knockout mutants can grow on butyrate in the absence of CO_2 when alternative electron acceptors ("auxiliary oxidants"), such as NO_3^-, trimethylamine-N-oxide, and dimethyl sulfoxide (DMSO), are provided (60, 103).

In PNSB, carboxylation-dependent redox balancing seems to be a general principle, because CO_2 fixation through RubisCO and the CBB cycle was shown to be important even for substrates (e.g., fumarate, succinate, malate, acetate) that are more oxidized than is the average case for cell carbon. However, in contrast to butyrate assimilation, no external CO_2 has to be added for photoheterotrophic growth on these substrates (49, 57). The results of recent flux analyses of *R. palustris* demonstrated that, during oxidation of these substrates, between 20% (fumarate) and 70% (acetate) of the CO_2 produced is refixed by RubisCO (84). Interestingly, redox balancing seems not to be limited to RubisCO and the CBB cycle. The ethylmalonyl-CoA pathway that uses reductive CO_2 fixation (via CCR) has recently been shown to serve as electron sink in photoheterotrophic acetate assimilation of *R. sphaeroides* (76),

and C_3-carboxylating enzymes have been suggested to be part of redox balancing in succinate-producing fermentative bacteria (86).

CARBOXYLASES IN SYNTHETIC MICROBIAL PATHWAYS

Improving autotrophic CO_2 fixation pathways by synthetic biology. The idea of improving CO_2 fixation pathways in autotrophic organisms to increase atmospheric CO_2 uptake and, hence, biomass production has been put forward in recent years (12, 78, 101). Traditionally, these efforts are focused on the optimization of existing natural pathways (e.g., the CBB cycle) and their corresponding carboxylases (e.g., RubisCO) that usually limit carbon flux (101). In contrast to such classical biotechnological efforts, the emerging field of synthetic biology provides an alternative approach to address this question. Here, completely novel pathways of improved functionality are intended to be created by the combination of different enzymatic reactions from different sources or organisms (36).

Synthetic biology could be used to create novel "customized" CO_2 fixation pathways that overcome any historical and evolutionary burden of the six natural autotrophic pathways. Such synthetic CO_2 fixation pathways could find applications in biotechnology and "green" chemistry (e.g., the production of biomass, biopharmaceuticals, or fine chemicals from CO_2 and an appropriate energy source) or in environmental protection (e.g., customized control of CO_2 emissions). Indeed, first projects have been launched that are funded by the Advanced Research Projects Agency-Energy (ARPA-E) and focus on the production of biofuels from CO_2 via novel biosynthetic routes (3). Customized CO_2 fixation pathways could also become an important tool in light of upcoming emission-trading schemes (EMTS), such as the European Union EMTS that was launched in 2005 to restrict and reduce the industrial emission of greenhouse gases (34). It is conceivable that, in the future, companies might use synthetic CO_2-fixing processes to balance their CO_2 emission levels.

Although several groups and companies have started exploring synthetic CO_2 fixation pathways, the practical application of completely novel autotrophic routes is limited by the availability of novel carboxylases. This need for novel carboxylating enzymes could be overcome by two different strategies. One strategy might involve the use of nonautotrophic carboxylases (e.g., anaplerotic, assimilatory, or biosynthetic carboxylases) in autotrophic CO_2 fixation. Such a physiological "reprogramming" was suggested recently for pyruvate carboxylase (12). The other strategy might rely on a (rational) design of novel carboxylating enzymes, e.g., "enoate carboxylases," which could be derived from ordinary enoate reductases, as proposed recently (42). Another example of such a rational approach is the screening of the active site of pyruvate:ferredoxin reductase for new (de)carboxylating reactions (9). It should be remembered that carboxylases have an important impact on the energetic costs and the physiology of an autotrophic pathway, so the use of novel carboxylases in synthetic biology has to be considered carefully.

Carboxylases in combinatorial biosynthesis. Many of the extender units used in polyketide biosynthesis are provided by carboxylating enzymes (see above). Consequently, carboxylases could become important tools in combinatorial biosynthesis aimed at creating novel "hybrid" antibiotics (26, 123). Introduction of additional carboxylase genes into natural biosynthetic gene clusters or expanding the substrate spectrum of existing carboxylases might ultimately provide novel extender units and thus yield novel secondary metabolites. This principle has been already successfully demonstrated. Introduction of the CCR gene into the erythromycin producer *Saccharopolyspora erythraea* resulted in the production of an ethyl-substituted erythromycin derivative (113). Similarly, CCR was shown to provide ethylmalonyl-CoA for an engineered carboxymethylproline synthase *in vitro*, yielding new precursors for carbapenem antibiotic synthesis (58). In addition, some CCR homologs show relaxed substrate specificities (42, 80, 130), which makes these enzymes attractive for future use in combinatorial biosynthesis.

Carboxylases in classical and synthetic biotechnological applications. In classical biotechnological applications, anaplerotic carboxylases serve as targets to redirect carbon flux to the synthesis of fermentation products that are derived from the TCA cycle. Pyruvate carboxylase of *Corynebacterium glutamicum* was shown to be the limiting enzyme (i.e., "bottleneck") in lysine fermentation. Overexpression of this enzyme resulted in a 50% increase in lysine production (91). Similarly, succinic acid production from glucose in *Escherichia coli* was shown to be increased by a factor of 3.5 by overexpression of PEP-carboxylase (87). Recently, the idea of the use of carboxylases in synthetic biotechnological applications has also been put forward. Novel synthetic routes that involve carboxylation reactions have been suggested for the production of 3-hydroxyisobutyric acid, a sustainable possible future building block in polymer chemistry (83, 95).

CONCLUDING REMARKS

In summary, carboxylases cover many different biological functions that extend beyond the simple fixation of CO_2 in autotrophic organisms to supply carbon for growth. Why is the principle of carboxylation so widely used in nature for such diverse functions as assimilation, anaplerosis, biosynthesis, and redox homeostasis? One reason might be that carboxylation provides an effective and simple way to extend a carbon substrate by a single C_1 unit. CO_2 is a moderately good electrophile but is far less toxic than formaldehyde, the alternative C_1 unit used in biology that requires even more "complicated" (tetrahydrofolate-)cofactor biochemistry (8, 62, 81, 125). Another reason might be that CO_2 is readily available to the cell, either as atmospheric gas or—more commonly—in its hydrated form (HCO_3^-), which is the substrate for all biotin-dependent carboxylases. This holds even truer when we assume that most of the CO_2 fixed in nonautotrophic pathways could be released directly inside the cell from oxidative processes (such as, e.g., the TCA cycle). Last, some carboxylases are able to serve multiple functions in different pathways, suggesting a physiological "reprogramming" of these enzymes during evolution. This is exemplified best by acetyl-CoA/propionyl-CoA carboxylase, which is the key enzyme in the autotrophic CO_2 fixation pathways of many archaea, whereas in most bacteria, homologs of this enzyme serve mainly in assimilation and/or biosynthesis. Even more notably, in the case of

C. aurantiacus, acetyl-CoA/propionyl-CoA carboxylase apparently fulfils all three functions at the same time (133).

How many new carboxylases of novel functionality are expected? A number of novel carboxylating enzymes with novel mechanisms have been described recently (21, 42, 70, 106). Thus, it can be expected that even more such enzymes will be discovered. Most likely, these are enzymes that function in substrate activation for assimilatory purposes (e.g., anaerobic degradation of recalcitrant substrates) or for biosynthesis (e.g., supply of unusual polyketide extender units). Clearly, we just have begun to understand some of the physiological functions (e.g., redox balancing) of carboxylases, and it would not be surprising if we could ascribe more functions (e.g., pH homoeostasis) to this versatile enzyme class.

Finally, the problem of steadily increasing atmospheric CO_2 concentrations requires society to find novel creative ideas to control this greenhouse gas. Customized (synthetic) CO_2 fixation pathways that allow the transformation of CO_2 into valuable compounds could become an important tool in challenging this globally significant question. Thus, research on CO_2-fixing enzymes would be of fundamental interest for the scientific community but would also receive attention from industry and society.

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