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REVIEW PAPER

# A survey of carbon fixation pathways through a quantitative lens

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## Abstract

**While the reductive pentose phosphate cycle is responsible for the fixation of most of the carbon in the biosphere, it has several natural substitutes. In fact, due to the characterization of three new carbon fixation pathways in the last decade, the diversity of known metabolic solutions for autotrophic growth has doubled. In this review, the different pathways are analysed and compared according to various criteria, trying to connect each of the different metabolic alternatives to suitable environments or metabolic goals. The different roles of carbon fixation are discussed; in addition to sustaining autotrophic growth it can also be used for energy conservation and as an electron sink for the recycling of reduced electron carriers. Our main focus in this review is on thermodynamic and kinetic aspects, including thermodynamically challenging reactions, the ATP requirement of each pathway, energetic constraints on carbon fixation, and factors that are expected to limit the rate of the pathways. Finally, possible metabolic structures of yet unknown carbon fixation pathways are suggested and discussed.**

**Key words:** ATP requirement, Calvin cycle, carbon fixation, electron sink, kinetics, metabolic pathways, oxygen sensitivity, reduction potential, reductive metabolism, thermodynamics.

## Introduction

Carbon fixation is one of the dominant biochemical processes in the biosphere, supplying the carbon building blocks for all living organisms. It became clear decades ago that, apart from the ubiquitous reductive pentose phosphate cycle (Bassham *et al.*, 1950), prokaryotic alternatives for carbon fixation also exist (Evans *et al.*, 1966; Fuchs, 1985; Ljungdahl, 1986). In recent years, these alternatives have started to accumulate at a staggering pace (Herter *et al.*, 2002*b*; Berg *et al.*, 2007; Huber *et al.*, 2008) and have already received some excellent reviews (Berg *et al.*, 2010*a*; Sato and Atomi, 2010; Berg, 2011; Fuchs, 2011; Hugler and Sievert, 2011). In this review, the carbon fixation pathways are compared according to various criteria and the metabolic aims they serve are analyzed. Specifically, in addition to sustaining autotrophic growth, they can be used for energy conservation and for the recycling of reduced electron carriers.

The structure and enzymatic components of each of the carbon fixation pathways, apart from the reductive pentose phosphate cycle that has been reviewed extensively (Stitt and Schulze, 1994; Poolman *et al.*, 2000; Raines, 2003, 2006; Peterhansel *et al.*, 2008), are given in Figs 1 and 2 and described in Table 1 and also in Supplementary Tables S1 and S2 at *JXB* online.

The carbon fixation pathways can be divided into two general categories. One category contains pathways (e.g. the reductive acetyl-CoA pathway) that directly utilize reduced C1 compounds (formic acid, formaldehyde, etc.) as free metabolites or as moieties attached to specific C1 carrier compounds (THF, MPT etc.) (Fig. 2). The other category contains pathways (e.g. the reductive TCA cycle) in which $CO_2$ is assimilated into the carbon backbone of other metabolites, such that no reduced C1 compounds (free or bound to specific carriers) are part of the pathway (Fig. 1).

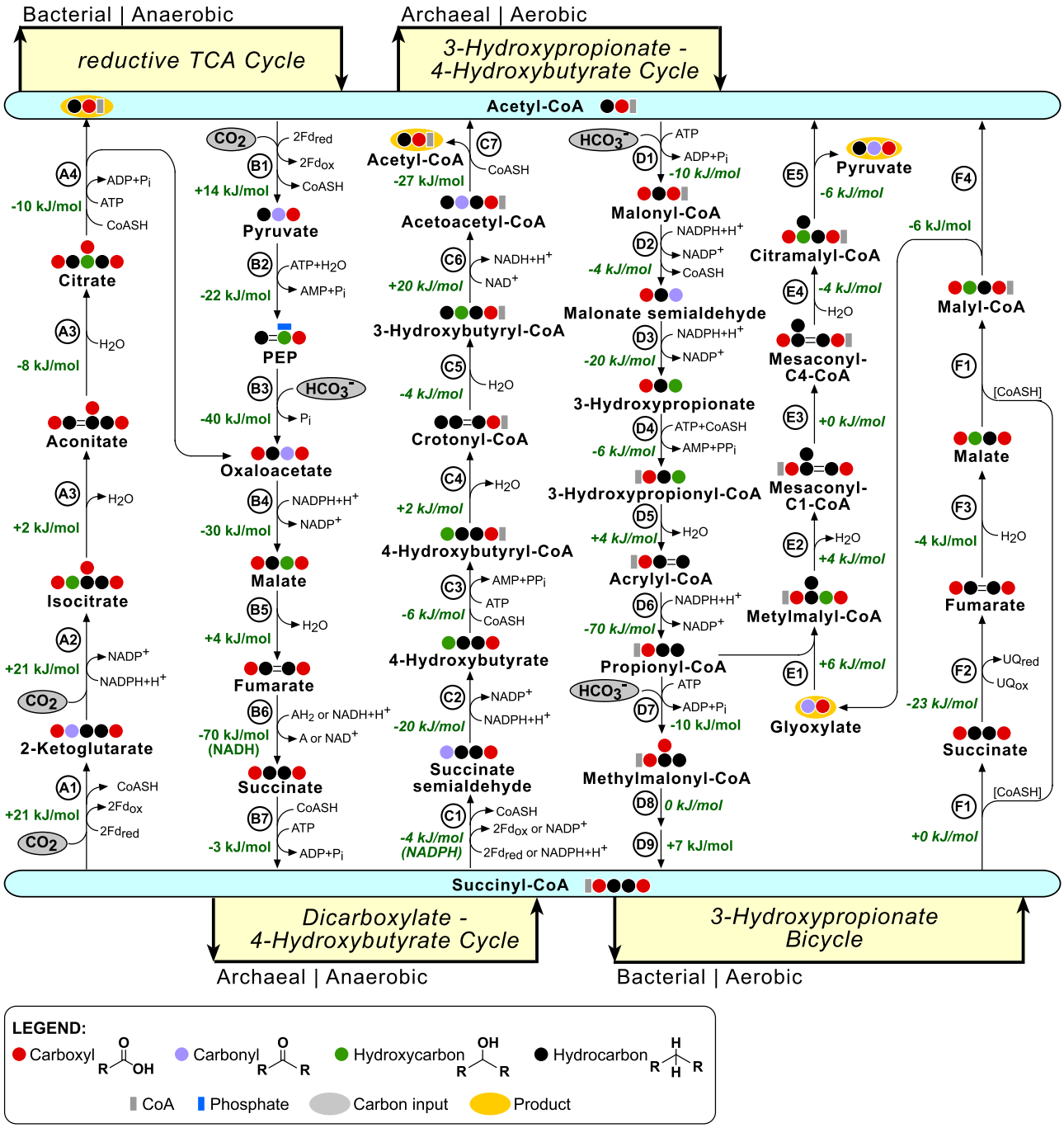


**Fig. 1.** The acetyl-CoA-succinyl-CoA carbon fixation cycles. Every circle corresponds to a substituted carbon atom. A colour notation scheme has been used to display the different functional groups composing the metabolites, which also corresponds to the oxidation states of the carbons: red indicates a carboxyl, purple corresponds to a carbonyl, green to a hydroxylcarbon, and black to a hydrocarbon. Fd corresponds to ferredoxin and UQ to ubiquinone. '$AH_2$' corresponds to a reduced electron donor, while 'A' represents an oxidized electron donor. Basic properties of the enzymes are given in Supplementary Table S1 at *JXB* online. Changes in Gibbs energy, $\Delta G_r$', at pH 7, ionic strength of 0.1 M, and reactant concentrations of 1 mM are shown in green and were calculated using eQuilibrator (http://equilibrator.weizmann.ac.il; Flamholz *et al.*, 2011) and available experimental Gibbs energies of formation, $\Delta G_f$', from (Alberty, 2003). $\Delta G_r$' values given in italics correspond to reactions in which $\Delta Gf_f$' of at least one reactant was not available and hence all $\Delta G_f$' values were calculated using a group contribution method (Jankowski *et al.*, 2008). Enzymes: (A1) 2-ketoglutarate synthase; (A2) isocitrate dehydrogenase; (A3) aconitase; (A4) ATP citrate lyase; (B1) pyruvate synthase; (B2) pyruvate water dikinase; (B3) PEP carboxylase; (B4) malate dehydrogenase; (B5) fumarase; (B6) fumarate reductase; (B7) succinyl-CoA synthetase; (C1) succinyl-CoA reductase; (C2) 4-hydroxybutyrate dehydrogenase; (C3) 4-hydroxybutyryl-CoA synthetase; (C4) 4-hydroxybutyryl-CoA dehydratase; (C5) enoyl-CoA hydratase (crotonase); (C6) 3-hydroxybutyryl-CoA dehydrogenase; (C7) acetyl-CoA C-acyltransferase; (D1) acetyl-CoA carboxylase; (D2) 3-oxopropionate dehydrogenase (malonyl-CoA reductase); (D3) 3-hydroxypropionate dehydrogenase; (D4)

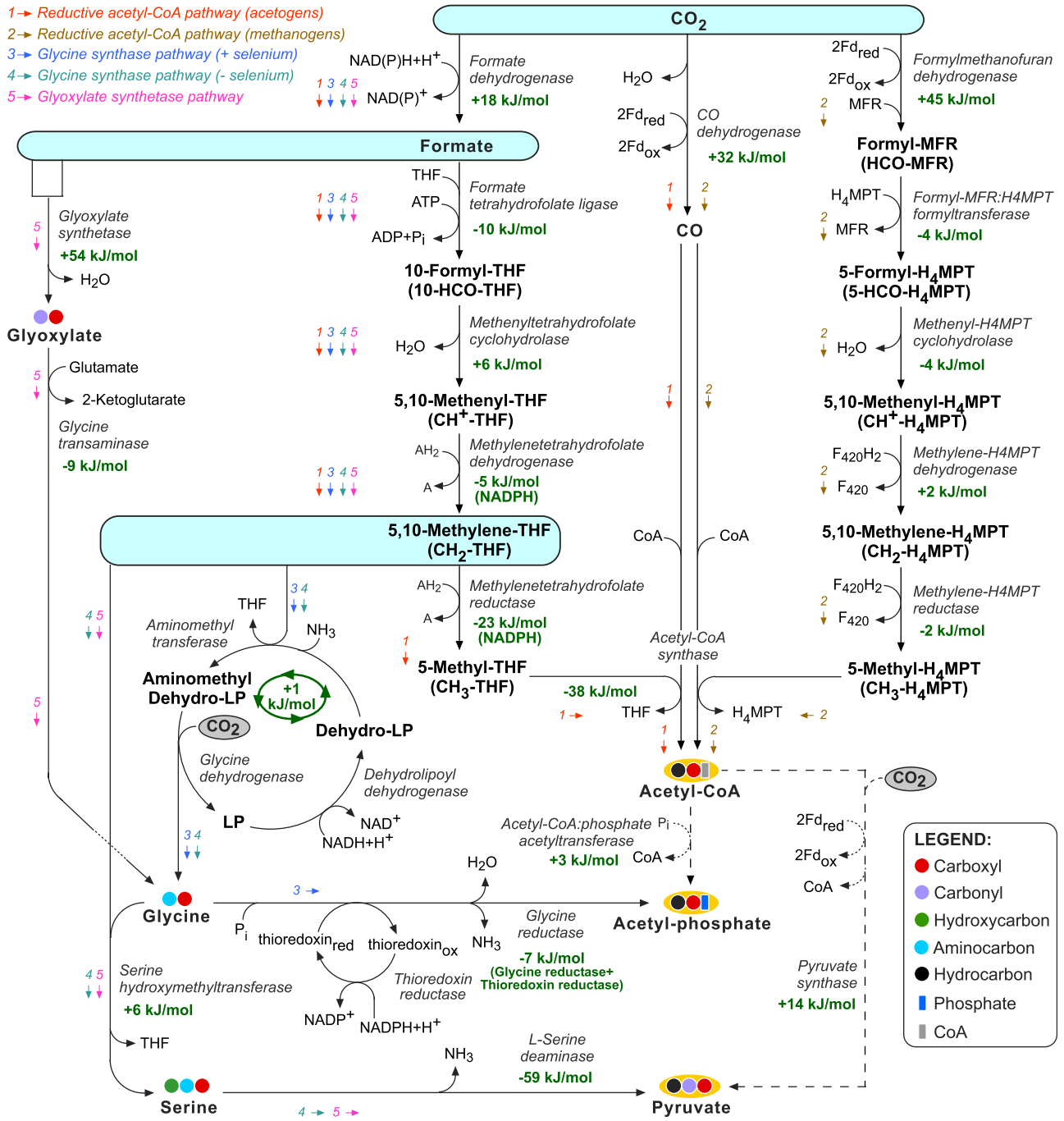


**Fig. 2.** The C1 carbon fixation pathways. Colouring and symbols are as in Fig. 1. THF corresponds to tetrahydrofolate, MPT to methanopterin, MFR to methanofuran, and $F_{420}$ to reduced deazaflavin factor 420. The glycine cleavage system is composed of the enzymes glycine dehydrogenase, aminomethyltransferase, and dehydrolipoyl dehydrogenase. 'LP' corresponds to lipoyl-protein (H-protein of the glycine cleavage system). Basic properties of the enzymes are given in Supplementary Table S2 at *JXB* online. Changes in Gibbs energy, $\Delta G_r'$, at pH 7, ionic strength of 0.1 M, and reactant concentrations of 1 mM are shown in green and were calculated using eQuilibrator (http://equilibrator.weizmann.ac.il; Flamholz *et al*., 2011) and available experimental Gibbs energies of formation, $\Delta G_f'$, from Alberty (2003). Changes in Gibbs energy for reactions involving THF, MFR, and MPT were taken from Maden (2000). Change in Gibbs energy for the glycine synthase system is taken from Liegel (1985).

3-hydroxypropionyl-CoA synthetase; (D5) 3-hydroxypropionyl-CoA dehydratase; (D6) acrylyl-CoA reductase; (D7) propionyl-CoA carboxylase; (D8) methylmalonyl-CoA epimerase; (D9) methylmalonyl-CoA mutase; (E1) methylmalyl-CoA lyase; (E2) methylmalyl-CoA dehydratase; (E3) mesaconyl-CoA C1-C4 CoA transferase; (E4) mesaconyl-C4-CoA hydratase; (E5) citramalyl-CoA lyase; (F1) succinyl-CoA:malate CoA transferase; (F2) succinate dehydrogenase; (F3) fumarase, and (F4) malyl-CoA lyase.

The C1 carbon fixation pathways are sometimes called 'linear' while the others are called 'cyclic'. However, this distinction can be problematic since the C1 carriers participating in the C1 carbon fixation pathways undergo a cyclic recycling similar to the recycling of the metabolites participating in the 'cyclic' carbon fixation pathways.

Apart from the reductive pentose phosphate cycle, all other non-C1 carbon fixation pathways share a common structural feature, as shown in Fig. 1. These cycles employ two conserved metabolites: acetyl-CoA and succinyl-CoA. Each cycle is composed of a 'downward arm', carboxylating acetyl-CoA to succinyl-CoA and an 'upward arm', regenerating acetyl-CoA and providing the cell with a fixed organic compound. Interestingly, each of the cycles shares at least one arm with another cycle, making the representation of the entire enzymatic scheme highly compact (Fig. 1).

While the four acetyl-CoA-succinyl-CoA pathways show a remarkable structural similarity, their phylogenetic distribution is very different (Table 1). The reductive TCA cycle (Buchanan and Arnon, 1990) and the 3-hydroxypropionate bicycle (Berg *et al.*, 2010*a*; Berg, 2011; Fuchs, 2011) are known to operate solely in bacterial lineages while the dicarboxylate-4-hydroxybutyrate cycle (Huber *et al.*, 2008) and the 3-hydroxypropionate-4-hydroxybutyrate cycle (Berg *et al.*, 2007; Berg *et al.*, 2010*b*; Walker *et al.*, 2010; Pratscher *et al.*, 2011) are used only by archaea (Table 1). Also, while the 3-hydroxypropionate bicycle and the 3-hydroxypropionate-4-hydroxybutyrate cycle overlap considerably, they do not share a common origin but rather have evolved independently (Berg *et al.*, 2010*a*; Berg, 2011; Fuchs, 2011).

In the C1 carbon fixation pathways, $CO_2$ molecules are reduced and attached to specific C1 carriers. Tetrahydrofolate (THF) is the primary carrier of reduced C1 fragments in most cells, serving an essential metabolic role in the biosynthesis of many central metabolites (Maden, 2000). The reductive acetyl-CoA pathway, the most widely spread C1 carbon fixation pathway (Table 1), utilizes this ubiquitous C1 carrier to enable $CO_2$ reduction and metabolism, as shown in Fig. 2, pathway 1 (Ljungdahl, 1986; Drake *et al.*, 2006, 2008; Ragsdale, 2008; Ragsdale and Pierce, 2008). The pathway uses the conserved CO-dehydrogenase-acetyl-CoA synthase complex to react the reduced C1 moiety with CoA and with another reduced $CO_2$ molecule (reduced to carbon monoxide) to generate acetyl-CoA (overall reaction: 5-methyl-THF+$CO_2$+CoA+2 ferredoxin(red)→acetyl-CoA+THF+2 ferredoxin(ox), Fig. 2). Methanogenic archaea use a distinct version of the reductive acetyl-CoA pathway, as shown in Fig. 2, pathway 2. Instead of THF and NAD(P)H, these archaea use unique C1 and redox carriers, such as methanopterin, methanofuran, and reduced deazaflavin factor 420 ($F_{420}$) (Keltjens *et al.*, 1983; Escalante-Semerena *et al.*, 1984*a*, *b*; DiMarco *et al.*, 1990).

The glycine synthase pathway, shown in Fig. 2, pathways 3 and 4, is not used for autotrophic growth. Instead, $CO_2$ fixation through this pathway serves only as an electron sink to recycle reduced electron carriers that are generated during the fermentation of purines and amino acids (Waber and Wood, 1979; Durre and Andreesen, 1982; Durre *et al.*, 1983; Schiefer-Ullrich *et al.*, 1984; Fuchs, 1985; Schneeberger *et al.*, 1999; Fonknechten *et al.*, 2010). The pathway recycles the electron carriers by reducing two $CO_2$ molecules to glycine, which is then converted to acetate and secreted from the cell. The glycine cleavage system, the core of the glycine synthase pathway, is a multi-protein complex that occurs throughout the tree of life and catalyses the reversible synthesis of glycine (overall reaction: 5,10-methylene-THF+$CO_2$+$NH_3$+NADH→glycine+THF+$NAD^+$; Fig. 2) (Kikuchi, 1973; Liegel, 1985; Pasternack *et al.*, 1992; Zhang and Wiskich, 1995). Glycine produced via the glycine cleavage system is converted to acetyl-phosphate via the selenoenzyme glycine reductase (Fig. 2, pathway 3) (Andreesen, 2004), or, when selenium is absent, to serine and pyruvate (Fig. 2, pathway 4) (Vogels and Van der Drift, 1976; Waber and Wood, 1979; Fuchs, 1985).

The last C1 carbon fixation pathway discussed here is an elusive pathway that was studied by a single research group. The glyoxylate synthetase pathway, shown in Fig. 2, pathway 5, was suggested to operate in the chloroplast of greening potato tubers and to be responsible for the reduction of three $CO_2$ molecules to pyruvate, which is then metabolized to the toxic alkaloid solanidine (Ramaswamy *et al.*, 1976; Ramaswamy and Sangeeta, 1983; Ramaswamy and Nair, 1984; Janave *et al.*, 1993, 1999). However, the gene encoding for glyoxylate synthetase, the central enzyme of the pathway that condenses two formate molecules into one glyoxylate molecule, is unknown. Since the enzyme has not been assigned to a gene and the reaction it catalyses was never found to operate in any other organism, the significance of this pathway remains unclear.

### *The carbon fixation pathways serve multiple metabolic goals*

One major functional difference between the C1 carbon fixation pathways and the others is that the former pathways are used for numerous metabolic goals, including energy conservation and the recycling of reduced electron carriers. By contrast, the non-C1 carbon fixation pathways are utilized solely for autotrophic growth. The only exception is the reductive pentose phosphate cycle that may serve, in some cases, solely as an electron sink for the recycling of electron carriers (Wang *et al.*, 1993; Tichi and Tabita, 2000; Joshi *et al.*, 2009).

A good example for multi-functionality of a carbon fixation pathway is the reductive acetyl-CoA pathway (Fig. 2, pathway 1) which is employed both heterotrophically and autotrophically (Drake *et al.*, 2006, 2008; Ragsdale, 2008; Ragsdale and Pierce, 2008). The acetogenic bacterium *Moorella thermoacetica*, for example, can use this pathway to convert glucose into three molecules of acetate (Fontaine *et al.*, 1942; Collins *et al.*, 1994): two acetate molecules are formed directly from the glycolytic fermentation of glucose while the third is produced by the reductive acetyl-CoA pathway, which recycles the reducing equivalents produced in the fermentation process. Notably, this

**Table 1.** Properties of the carbon fixation pathways

| Pathway | Distribution | Resources need for the synthesis of pyruvate | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Electron donors | | | Electron acceptors | ATP requirement[a] | Carbon species | |
| | | Ferredoxin pairs | NAD(P)H | Others | | | $CO_2$ | $HCO_3$ |
| Reductive pentose phosphate cycle | The Plantae and Protista kingdoms, cyanobacteria and members of the α, β and γ subdivisions of proteobacteria (photosynthetic & chemolithotrophic) | – | 5 | – | – | 7 | 3 | – |
| Reductive TCA cycle | Green sulphur bacteria (*Chlorobi*), members of the thermophilic bacterial phylum *Aquificae*, members of the *Nitrospira* phylum and members of the α, δ, and ϵ subdivisions of proteobacteria (photosynthetic & chemolithotrophic) | 2 | 3 | – | – | 2 | 3 | – |
| Dicarboxylate-4-hydroxybutyrate Cycle | Anaerobic or microaerobic thermophilic archaea orders of *Desulfurococcales* and *Thermoproteales* (chemolithotrophic) | 2 or 3 | 2 or 3 | 1 | 1 $NAD^+$ | 5 | 2 | 1 |
| 3-Hydroxypropionate-4-hydroxybutyrate cycle | Aerobic thermophilic archaea of the order *Sulfolobales* and possibly the mesophilic*Thaumarchaeota* phylum (chemolithotrophic) | – | 7 | – | 1 Ubiquione + 1 $NAD^+$ | 9 | – | 3 |
| 3-Hydroxypropionate bicycle | The green non-sulphur bacterium *Chloroflexus aurantiacus* and related *Chloroflexi* (photosynthetic) | – | 6 | – | 1 Ubiquione | 7 | – | 3 |
| Reductive acetyl-CoA pathway (acetogens) | Mostly members of the bacterial class of *Clostridia* but also other members of the *Firmicutes* and *Planctomycetes* phylums (chemolithotrophic) | 2 | 3 | – | – | <1 | 3 | – |
| Reductive acetyl-CoA pathway (methanogens) | Methnogens and other archaea of the *Euryarchaeota* phylum (chemolithotrophic) | 3 | – | 2 $F_{420}H_2$ | – | <1 | 3 | – |
| Glycine synthase pathway (glycine reductase) | Members of the bacterial class of *Clostridia* (heterotrophic) | 1 | 4 | – | – | 1 | 3 | – |
| Glycine synthase pathway (serine hydroxymethytransferase) | Members of the bacterial class of *Clostridia* (heterotrophic) | – | 5 | – | – | 2 | 3 | – |
| Glyoxylate synthetase pathway | The chloroplast of greening potato tubers (photosynthetic) | – | 5 | – | – | 1 | 3 | – |

[a] Assuming pyrophosphate is hydrolysed into two phosphates.

metabolic alternative enables the production of four ATP molecules instead of the two normally produced in glycolysis. Usually, in order to recycle the reduced electron carriers, acetyl-CoA molecules formed in glycolysis are reduced to acetaldehyde and then to ethanol. However, in heterotrophic organisms that use the reductive acetyl-CoA pathway, the reduced electron carriers are recycled through carbon fixation, freeing acetyl-CoA molecules to be converted into acetyl-phosphate. Acetyl-phosphate then transfers its phosphate groups to ADP, forming acetate and ATP and thereby increasing the ATP yield by two extra molecules per glucose (Drake, 1994). This route represents the highest known ATP yield in fermentation (Drake *et al.*, 2006).

The reductive acetyl-CoA pathway is also the only autotrophic carbon fixation pathway that can be used for energy conservation. The product of the pathway, acetyl-CoA, can be converted to acetate via acetyl-phosphate and provide an ATP that recoups the ATP-dependent linkage of formate and tetrahydrofolate (Fig. 2). In addition, the electron transport in one or more of the pathway's oxidation–reduction reactions (probably methylenetetrahydrofolate

reductase) is coupled to sodium translocation across the membrane, which supports the formation of ATP via ATPases (Imkamp and Muller, 2007; Ragsdale and Pierce, 2008; Ljungdahl, 2009; Biegel and Muller, 2010; Fuchs, 2011; Martin, 2011). Consequently, the product of the pathway, acetyl-CoA, can either be directed to acetate and energy conservation or to pyruvate and biomass production (Fig. 2).

Notably, the reductive acetyl-CoA pathway and the glycine synthase pathway are quite similar in their general reaction sequence: (1) $CO_2$ is reduced and attached to a C1 carrier (THF or MPT); (2) the attached C1 moiety is further reduced; (3) a complex (CO-dehydrogenase-acetyl-CoA synthase or glycine synthase) reacts the reduced C1 moiety with another $CO_2$ molecule to give an energized C2 compound (acetyl-CoA or glycine); (4) the energized C2 compound is converted into acetyl-phosphate; (5a) the acetyl phosphate is converted to acetate while conserving energy in the form of ATP, thereby recouping the ATP invested at the beginning of the pathway; (5b) alternatively, biomass can be produced by converting the energized C2 compound into pyruvate.

Despite this apparent similarity, the usage of the reductive acetyl-CoA pathway is much more versatile than that of the glycine synthase pathway. The reductive acetyl-CoA pathway is used for autotrophic growth, energy conservation, and as an electron sink. However, the glycine synthase pathway serves only as an electron sink. Energy conservation is probably not possible through the glycine synthase pathway since it does not utilize the enzyme methylenetetrahydrofolate reductase which is thought to be coupled to sodium translocation across the membrane and energy conservation (Martin, 2011). However, there is no clear reason for why this pathway cannot be used for autotrophic growth.

### *The carbon fixation pathways differ in their oxygen tolerance*

A major difference between the different acetyl-CoA-succinyl-CoA pathways is their suitability for autotrophic growth in aerobic environments. Pathways which employ oxygen-sensitive enzymes or cofactors are expected to operate only in organisms that occupy anaerobic environments. The oxygen-sensitive enzymes of the acetyl-CoA-succinyl-CoA pathways are mainly 2-ketoglutarate synthase (enzyme 'A1' in Fig. 1), pyruvate synthase (enzyme 'B1'), and 4-hydroxybutyryl-CoA dehydratase (enzyme 'C4'), all contain an iron-sulphur cluster and involve free radical intermediates (Kerscher and Oesterhelt, 1981; Ragsdale, 2003; Martins *et al.*, 2004; Scott *et al.*, 2004; Naser *et al.*, 2005; Imlay, 2006). Hence, organisms that use the reductive TCA cycle and the dicarboxylate-4-hydroxybutyrate cycle are restricted to anaerobic environments, while the 3-hydroxypropionate bicycle can operate under aerobic conditions (Fig. 1).

However, the oxygen sensitivity of specific enzymes can vary greatly between organisms, making a clear distinction between aerobic and anaerobic pathways problematic. For example, the 4-hydroxybutyryl-CoA dehydratase enzyme of organisms which employ the 3-hydroxypropionate-4-hydroxybutyrate cycle is oxygen tolerant (Berg *et al.*, 2010*b*), enabling these organisms to grow in aerobic environments. To support the aerobic operation of this cycle further, it uses a NADPH-dependent rather than a ferredoxin-dependent succinyl-CoA reductase (enzyme 'C1') (Berg *et al.*, 2007, 2010*b*; Ramos-Vera *et al.*, 2011) and it does not use pyruvate synthase to convert acetyl-CoA to a C3 compound for gluconeogenesis. Instead, succinyl-CoA is diverted from the cycle and is oxidized to malate and oxaloacetate, which then undergo decarboxylation to pyruvate and PEP (Estelmann *et al.*, 2011; Fuchs, 2011).

Indeed, while members of the anaerobic archaeal orders of Desulfurococcales and Thermoproteales operate the dicarboxylate-4-hydroxybutyrate cycle, members of the aerobic archaeal order of Sulfolobales employ the 3-hydroxypropionate-4-hydroxybutyrate cycle, as expected by the oxygen tolerance of each of the pathways. However, even this distinction does not hold in all cases: it was found that even aerobic members of Desulfurococcales operate the dicarboxylate-4-hydroxybutyrate cycle (although at low $O_2$ concentrations) and anaerobic members of Sulfolobales operate the 3-hydroxypropionate-4-hydroxybutyrate cycle (Berg *et al.*, 2010*b*). This suggests that the identity of the carbon fixation pathway used might not always be optimized to environmental conditions, but might rather be a historical remnant of the evolutionary trajectory of a species.

Notably, even 2-ketoglutarate synthase and pyruvate synthase are not always oxygen sensitive. Pyruvate synthase isolated from the strictly anaerobic bacterium *Desulfovibrio africanus* is highly stable in the presence of oxygen (Pieulle *et al.*, 1997), suggesting that organisms that operate the reductive TCA cycle might be able to adapt to aerobic conditions. Indeed, *Hydrogenobacter thermophilus*, *Aquifex pyrophilus*, and other *Aquificae* bacteria grow aerobically using the reductive TCA cycle (Shiba *et al.*, 1985; Beh *et al.*, 1993; Hugler *et al.*, 2007). *Hydrogenobacter thermophilus*, for example, uses two isozymes of 2-ketoglutarate synthase, one of which is relatively oxygen stable, enabling rapid growth using molecular oxygen as an electron acceptor (Yamamoto *et al.*, 2003, 2006).

Of the C1 carbon fixation pathways, the reductive acetyl-CoA pathways (Fig. 2, pathways 1 and 2) can only tolerate oxygen at a very low level (Drake *et al.*, 2006, 2008; Imkamp and Muller, 2007; Ragsdale, 2008; Ragsdale and Pierce, 2008). This is because the pathway employs two of the most oxygen sensitive enzymes known: NADPH-dependent formate dehydrogenase and the CO-dehydrogenase-acetyl-CoA synthase complex.

Interestingly, while the glycine synthase pathway is known to operate only in anaerobes, it can potentially support autotrophic growth under aerobic conditions. Instead of the NADPH-dependent, oxygen-sensitive formate dehydrogenase, an oxygen-tolerant, NADH-dependent enzyme can be used (Tishkov and Popov, 2006). All other enzymes required for pyruvate synthesis by the pathway are oxygen tolerant.

### *Thermodynamically challenging reactions*

Different carbon fixation pathways differ in their demand for cell resources (Table 1) (Berg, 2011; Berg *et al.*, 2010*a*; Boyle and Morgan, 2011; Fuchs, 2011; Hugler and Sievert, 2011). The resource requirements can be generally divided into two parts: the ATP and NADPH equivalents needed for the reduction and fixation of $CO_2$ and the carbon and nitrogen required for the enzymatic machinery carrying the fixation process. The former part mostly relates to the energetics of the carbon fixation process while the latter mostly refers to its kinetics—efficient enzymatic machinery requires less enzymes to sustain a desired flux, resulting in a lower burden of enzyme production. In the following three sections, the carbon fixation pathways are discussed and compared according to energetic aspects. In the three sections to follow, we deal with the kinetics of the pathways, discussing enzymes that are expected to limit the rate of carbon fixation.

Before comparing the carbon fixation pathways as a whole, it is worthwhile to examine some of the reactions that are expected to present a thermodynamic difficulty. Figures 1 and 2 detail $\Delta G_r$' values for all the reactions participating in the carbon fixation pathways, under pH 7, ionic strength of 0.1, and reactants (substrates and products) concentrations of 1 mM [$\Delta G_r$' values were calculated as described in Alberty (2003), Flamholz *et al.* (2011), and Jankowski *et al.* (2008)]. $\Delta G_r'^{o}$ values were not used since they correspond to non-physiological reactant concentrations of 1 M, whereas 1 mM is a 'more reasonable' estimate for metabolite concentrations *in vivo* (Weber, 2002; Henry *et al.*, 2006; Bennett *et al.*, 2009; Bar-Even *et al.*, 2011*a*). Exploring Figs 1 and 2, the reactions that present a considerable thermodynamic challenge are mostly oxidoreductase reactions. More specifically, the most energetically challenging reactions are some of the core $CO_2$ fixing reactions, including pyruvate and 2-ketoglutarate synthases (reactions 'A1' and 'B1' in Fig. 1), isocitrate dehydrogenase (reaction 'A2'), formate dehydrogenase, CO dehydrogenase and formylmethanofuran dehydrogenase (Fig. 2). Notably, most of these enzymes utilize the low reduction potential (i.e. highly energized) ferredoxin ($E'^{0}$= –430 mV) as an electron donor instead of NAD(P)H ($E'^{0}$= –320 mV). However, even with the extra energetic push of this electron donor, operating these reactions in the required direction is energetically challenging. How can cells overcome this energetic barrier?

First, the ferredoxin (and NADPH) pool in cells operating these reactions might be over-reduced ([ferredoxin$_{red}$]/[ferredoxin$_{ox}$] >>1). Mechanisms to maintain such over-reduction are discussed in detail in (Fuchs, 2011; Martin, 2011). However, the over-reduction of the electron donors might not suffice. For example, even if the vast majority of ferredoxin molecules are reduced, [ferredoxin$_{red}$]/[ferredoxin$_{ox}$]=100, $\Delta G_r$' will be lowered by only 11 kJ mol$^{-1}$ (compared with the 'standard' 1 mM metabolite concentration case), not enough for some of the challenging reactions.

A parallel solution is to keep the concentrations of the products much lower than that of the substrates. For example, keeping [acetyl-CoA] at ~1 mM and [pyruvate] and [CoA] at ~1 μM, while operating at high [$CO_2$] of 100 μM (not uncommon in anaerobic environments), will lower $\Delta G_r$' for pyruvate synthase by 28 kJ mol$^{-1}$, enabling the operation of the reaction in the carboxylation direction even without the over-reduction of the ferredoxin pool. The drawback of this approach is that keeping the products of some enzymes at a low concentration of ~1 μM, below the $K_M$ of most enzymes utilizing them (Bar-Even *et al.*, 2011*b*), will result in a low reaction rate of the enzymes which use these compounds as substrates. This trade-off between thermodynamics and kinetics means that this approach will probably not be applied to its full extent and hence some over-reduction of the electron carrier pool will be required.

Some thermodynamic barriers are so high, that both a significant over-reduction of the electron carriers and a large modulation of reactant concentrations are required. Examples for such thermodynamic barriers are the sequential operation of 2-ketoglutarate synthase and isocitrate dehydrogenase (reaction 'A1' and 'A2' in Fig. 1), which together presents a cumulative barrier of >40 kJ mol$^{-1}$, and the very positive $\Delta G_r'^{o}$ associated with CO dehydrogenase and formylmethanofuran dehydrogenase (Fig. 2).

Of special interest is the enzyme glyoxylate synthetase, which seems to present an insurmountable energetic challenge under physiological metabolite concentrations. Moreover, since this reaction follows formate dehydrogenase, another energetically challenging reaction, the energetic barrier for $CO_2$ assimilation into glyoxylate is extremely large ($\Delta G_r'^{o}$ >70 kJ mol$^{-1}$). In fact, to enable the operation of these reactions in tandem, even under an extremely high [$CO_2$] of 10 mM and [NAD(P)H]/[NAD(P)$^+$] of $10^6$, [glyoxylate] will have to be <1 nM. This concentration is too low to be of any metabolic significance. Our analysis therefore suggests that the glyoxylate synthetase pathway might be erroneous. Alternatively, it might be the case that glyoxylate synthetase is coupled to some exergonic reactions that push it forward. While no evidence for this has been found yet, the enzyme's complete mechanism is still unknown.

### *The ATP requirement of the different carbon fixation pathways varies greatly*

The number of reducing equivalents required for $CO_2$ fixation is identical for all carbon fixation pathways as they are the result of only the number of electrons in the starting and ending compounds. By contrast, the ATP requirement of different carbon fixation pathways varies greatly, as shown in Table 1. Why does the ATP requirement differ to such an extent between the different pathways (Berg *et al.*, 2010*a*; Berg, 2011; Fuchs, 2011; Hugler and Sievert, 2011)? Several possible, non-mutually exclusive explanations for this phenomenon are suggested and analysed here.

A common explanation relates to the identity of the electron donors. Different pathways employ different

combinations of NADPH and ferredoxins to donate electrons. When both ferredoxin and NAD(P)H are not over-reduced or over-oxidized, the reduction potential of ferredoxin is lower than that of NAD(P)H (i.e. is more energetic). This might indicate that pathways that use more ferredoxins (instead of NADPH) require less ATP since they get an extra energetic push from the electron donor. In fact, it was recently suggested that reduced ferredoxin can be regarded as a currency of energy just as ATP (Martin, 2011). However, it is important to note that the difference in reduction potentials between NADPH and ferredoxin usually provides an energetic push well below that of ATP. In fact, under standard conditions the replacement of NADPH by two ferredoxins translates to an extra energetic contribution of ~20 kJ $mol^{-1}$, while the hydrolysis of an ATP molecule, under physiological conditions, contributes over 50 kJ $mol^{-1}$ (Tran and Unden, 1998; Wackerhage *et al.*, 1998; Bennett *et al.*, 2009). Therefore, replacing NADPH by ferredoxin is unlikely to account fully for the significant differences in ATP requirements between the different pathways.

Another line of explanation suggests that the variability in ATP requirement relates to the differences in redox potential between the environments the pathways operate in. Specifically, it was proposed that, for chemolithotrophic growth, it takes more energy to produce the same biomass under oxic conditions than under anoxic conditions (McCollom and Amend, 2005). This is because the electrons that are needed to reduce $CO_2$ to the various organic constituents of the cell tend to be at higher energy in anoxic environments whose reduction potential is lower than that of oxic environments (McCollom and Amend, 2005). It has therefore been deduced that organisms that live in anaerobic environments should require less ATP for carbon fixation.

However, this reasoning is problematic. Electrons taken from the electron donors are not directly used to reduce $CO_2$ to the organic constituents of the cell. Rather, they are first stored in the cellular electron carriers, for example, NADPH and ferredoxin. Since the ATP requirement discussed here is invested in the electron path from the carriers to the fixed carbon (i.e. carbon fixation) and not in the upstream electron path (i.e. reduction of the electron carriers by the electron donors), changes in the reduction potential of the environment are not expected to have a significant effect on the energy required for carbon fixation. Instead, the reduction potential of the environment will mainly affect the way the cellular electron carriers are reduced. Organisms that grow chemolithotrophically and use electron donors with high reduction potential (e.g. $Fe^{2+}$) must use reverse electron flow to reduce the cellular electron carriers. In reverse electron flow, some of the energy generated by the flow of electrons from the donor to the terminal acceptor and stored in the proton (or sodium) gradient across the membrane is used to push electrons 'uphill' from the donor to NAD(P)H (or other electron carrier) (DiSpirito and Tuovinen, 1982; Brune, 1989; Stouthamer *et al.*, 1997; Elbehti *et al.*, 2000; Blankenship, 2002; Klamt *et al.*, 2008).

Unlike the reduction potential of the environment which can vary greatly, the reduction potential range of the cellular electron carriers is rather restricted. A reduction potential far from the standard reduction potential means that either the reduced form or the oxidized form must be at a very low concentration. This is deleterious for cell functions since it results in poor kinetics of the enzymes utilizing the compound. Considering NADPH, which participates in all carbon fixation pathways, as a benchmark, it is extremely unlikely that [NADPH]/[$NADP^+$] can be lower than $10^{-6}$ or higher than $10^6$. This limits the reduction potential available for carbon fixation to lie between –500 mV and –150 mV, regardless of the reduction potential of the environment.

Another potential explanation for the range of ATP requirements involves the energy available for different organisms to operate carbon fixation. Specifically, organisms that occupy environments that are poor in energy sources will employ one of the pathways with a low ATP requirement. For example, methanogens and acetogens operate very close to the thermodynamic limit, having little spare energy to invest (Deppenmeier and Müller, 2008; Fuchs, 2011). The extremely low energy requirement of the reductive acetyl-CoA pathway therefore fits their energetic constraints. The anaerobic photosynthetic green sulphur bacteria, which live at a depth of more than 100 m below the surface of anoxic water-bodies such as the Black Sea, provide another example (Overmann *et al.*, 1992; Manske *et al.*, 2005). Such an extremely low-light environment (<4 μE $m^{-2}$ $s^{-1}$) constrains the amount of energy the organism can spend on carbon fixation. Indeed, the green sulphur bacteria use the reductive TCA cycle, which requires only two ATP molecules to form pyruvate. This organism also uses photosystem I, which can directly reduce ferredoxin and NADPH without resorting to energy-consumptive reverse electron flow (Hauska *et al.*, 2001).

Finally, the inorganic carbon concentration is an additional environmental factor that has a significant effect on the ATP requirement. Higher [$CO_2$] translates to a more favourable energetics of carbon fixation and hence to a lower ATP requirement. For example, organisms that operate the reductive acetyl-CoA pathway cannot be cultivated without the addition of significant amounts of inorganic carbon (>130 mM) (Drake *et al.*, 2006). As for sustaining the electron carriers' reduction potential low enough by reverse electron flow, the cellular concentration of inorganic carbon can be increased by energy-dependent carbon-concentrating mechanisms which are known to operate in both chemolithotrophic (Heinhorst *et al.*, 2006; Yeates *et al.*, 2008; Dobrinski, 2009; Minic and Thongbam, 2011) and photosynthetic organisms (Kaplan and Reinhold, 1999; Giordano *et al.*, 2005; Price *et al.*, 2008).

### *Feasibility of carbon fixation as an interplay between various factors*

In the above analysis, three main factors that affect the energetic feasibility of carbon fixation were noted. These

are: the number of ATP molecules hydrolysed in the process, the cellular concentration of inorganic carbon, and the redox potential that is utilized to reduce $CO_2$. Ferredoxin and NADPH are expected to have different (concentration-dependent) reduction potentials under physiological conditions. Yet, for the ensuing analysis, the (concentration-dependent) reduction potentials of both ferredoxin and NADPH have been treated as identical. While such an assumption is certainly over-simplistic, it enables straightforward comparison of the different carbon fixation pathways and provides some key observations into the constraints imposed on carbon fixation.

Figure 3 presents the interplay between these parameters in the fixation of inorganic carbon to three central metabolites. For each product, the minimal number of ATP molecules required for thermodynamic feasibility is indicated. Maximal $CO_2$ concentration is taken to be 10 000× ambient, corresponding to pure $CO_2$ at about 3 atm. The electron carrier's reduction potential is taken to lie between –500 mV and –150 mV, as explained above. While other varying factors such as pH, ionic strength, and metal ion concentrations also affect the energetics of the carbon fixation process (Alberty, 2003; Bar-Even *et al.*, 2010), it was found that these typically have a secondary effect when compared with the three parameters analysed in Fig. 3.

Comparing Fig. 3 with Table 1 suggests that most carbon fixation pathways hydrolyse more ATP molecules than are needed for energetic feasibility. For example, the reductive pentose phosphate cycle, which produces glyceraldehyde-3-phosphate, consumes 9 ATP molecules even though the thermodynamic minimal requirement is predicted at 4–5 ATP molecules (given a redox potential of ~300 mV and an ambient $CO_2$ concentration). The extra ATP molecules are not essential for the thermodynamic feasibility but are used to create irreversibility points that ensure the cycle's operation in the correct direction (Bar-Even *et al.*, 2010) and also to establish a strong chemical motive force that translates to an increased carbon fixation rate (Beard and Qian, 2007).

More interesting are those pathways that, according to Fig. 3, operate near their thermodynamic limit. The reductive TCA cycle requires two ATP molecules to reduce $CO_2$ to pyruvate. Therefore, in order to operate the cycle under moderate $CO_2$ concentrations, the host organism must keep the reduction potential available for carbon fixation well below –300 mV. The reductive acetyl-CoA pathway presents an even harsher energetic challenge. The reductive acetyl-CoA pathway hydrolyses less than one ATP molecule in the formation of pyruvate: one ATP is invested in the ATP-dependent linkage of formate and tetrahydrofolate (Fig. 2) but, in addition, ATP is produced later following coupling of some of the pathway's oxidation–reduction reactions to sodium translocation across the membrane (Imkamp and Muller, 2007; Ragsdale and Pierce, 2008; Ljungdahl, 2009; Biegel and Muller, 2010; Fuchs, 2011; Martin, 2011). Figure 3 suggests that this extremely low ATP investment enables carbon fixation only at a very low reduction potential and at very high inorganic carbon concentrations, as supported by the literature (Drake *et al.*, 2006).

Interestingly, while the glycine synthase pathway also displays a very low ATP requirement, it is less likely to run into energetic difficulties. As this pathway is used as an electron sink, reducing power can accumulate within the cell until the pathway becomes feasible. It is reasonable to suggest that organisms that employ the pathway operate at reduction potentials well below –300 mV, enabling them to use $CO_2$ as an electron sink even at near ambient concentrations.

### *Estimating the kinetics of carbon fixation*

While the energetics of carbon fixation can be calculated rather precisely, its kinetics is more difficult to estimate in

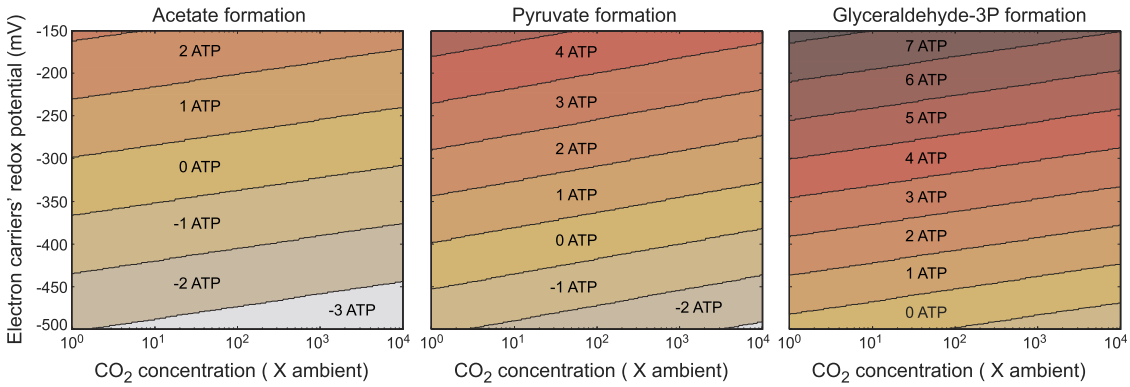


**Fig. 3.** ATP requirement for the reduction of $CO_2$ to acetate, pyruvate, and glyceraldehyde-3-phosphate, as a function of the electron carriers' redox potential and $CO_2$ concentration. The ATP requirement was calculated as: $\Delta G_r'$ ($n$ NADPH+m $CO_2 \rightarrow n$ $NADP^+$+product+$y$ $H_2O$)/$\Delta G_r'$ (ADP+$P_i$→ATP+$H_2O$), where Gibbs energies of formation were taken from Alberty (2003), [NADPH]/[$NADP^+$] was taken to match the cellular redox potential in the $y$-axis, [$CO_2$] was taken according to the $x$-axis (ambient conditions=387 ppm), [product]=1 μM and $\Delta G_r'$ (ADP+$P_i$→ATP+$H_2O$)=50 kJ $mol^{-1}$ as in *E. coli* (Bennett *et al.*, 2009). $\Delta G_r'$ was calculated at pH 7 and an ionic strength of 0.1 M using the methodology presented in Alberty (2003). If the calculated ATP requirement was not an integer, it was rounded up. Negative values mean the ATP can be produced, rather than consumed, by the pathway.

an accurate manner. One way to do so is by Metabolic Flux Analysis and similar methods that use mathematical modelling and experimental flux measurements to calculate the flux distribution within an entire biochemical reaction network (Morgan and Rhodes, 2002; Schwender *et al.*, 2004; Libourel and Shachar-Hill, 2008; Kruger and Ratcliffe, 2009). Such a recent study, for example, reconstructed the flux maps of photoautotrophic metabolism by a computational analysis of dynamic isotope labelling measurements (Young *et al.*, 2011). This methodology represents a promising tool for the elucidation of the *in vivo* kinetics of different carbon fixation pathways. However, experimental flux measurements are scarce, hence limiting the applicability of this approach.

In a previous study, a simplified but generic method to estimate the kinetics of a pathway was suggested based on a criterion termed the 'pathway specific activity' (Bar-Even *et al.*, 2010). The pathway specific activity is analogous to an enzyme's specific activity and is defined to be the maximal rate of product formation by 1 mg of pathway total protein (Bar-Even *et al.*, 2010). Briefly, in order to maintain a product flux of 1 μmol $min^{-1}$ through a given enzyme *i*, $1/V_i$ mg of that enzyme is needed, where $V_i$ is the enzyme's specific activity, in units of μmol $min^{-1}$ $mg^{-1}$. $1/V_i$ is therefore the enzyme cost of that reaction (mg enzyme needed to achieve 1 μmol $min^{-1}$ of flux). The enzyme cost of an entire linear pathway is the sum of the individual enzyme costs, i.e. $\Sigma(1/V_i)$. This sum corresponds to how many mg of total enzyme are needed to sustain an overall flux of 1 μmol $min^{-1}$. The pathway specific activity is defined to be the flux (μmol $min^{-1}$) sustained by 1 mg of pathway total protein; therefore, it equals the reciprocal of the total enzyme cost, $1/(\Sigma(1/V_i))$. In the general case of non-linear pathways, the fluxes through individual enzymes are not necessarily the same. In such a case, a stoichiometric coefficient, $w_i$, is assigned to each reaction *i*, which corresponds to the number of catalytic cycles the reaction requires to produce one molecule of the pathway's product. The enzyme cost for each reaction is multiplied by its stoichiometric coefficient and the pathway specific activity is given by $1/(\Sigma(w_i/V_i))$ (Bar-Even *et al.*, 2010).

The overall flux through a pathway is approximated by the pathway specific activity criterion when: (i) the enzymes are substrate saturated, (ii) the rate of the backward reaction of each enzyme is negligible compared with the rate of its forward reaction, and (iii) enzyme expression levels are balanced based on each enzyme's specific activity (no 'surplus' of any enzyme). Obviously, in natural pathways none of these requirements fully holds; therefore the pathway specific activity serves mainly as an upper limit useful for comparing the overall rate of alternative pathways realizations.

The pathway specific activity approximation has several further drawbacks. For example, the criterion is based on kinetic parameters measured *in vitro*, which can be considerably different from those experienced *in vivo* (Ringe and Petsko, 2008; Wright *et al.*, 1992). Also, for some enzymes, kinetic data are scarce or might be improved considerably if the enzyme could have been purified more efficiently. However, in spite of these shortcomings, the pathway specific activity approximation provides a useful, unbiased methodology to get a rough evaluation of the pathway kinetics and to compare alternative metabolic pathways when direct experimental measurements are not available.

### *Carboxylating enzymes are often among the slowest enzymes in the carbon fixation pathways*

Metabolic Control Analysis suggests that the flux within a pathway is controlled by several enzymes, such that no enzyme can be regarded as a sole kinetic bottleneck (Fell, 1992; Stitt, 2004). However, most carbon fixation pathways utilize one or more enzymes with especially slow kinetics. These enzymes are expected to be expressed at a high expression level and hence become a significant sink for the carbon and nitrogen resources of the cell, possibly limiting its growth. The utilization of such enzymes can dramatically decrease the pathway specific activity of the carbon fixation pathway.

A detailed quantitative analysis suggests that many of the enzymes that considerably lower the pathway specific activity of carbon fixation pathways are the carboxylating enzymes themselves (Bar-Even *et al.*, 2010). Kinetically superior carboxylating enzymes are those which display high $k_{cat}$ but also have good affinity towards $CO_2$ or $HCO_3^-$. As suggested previously (Bar-Even *et al.*, 2010), the most kinetically favourable carboxylating enzymes appear to be PEP and pyruvate carboxylases, having high specific activity (25–50 μmol $min^{-1}$ $mg^{-1}$) and a high affinity toward $HCO_3^-$.

Rubisco, the carboxylating enzyme that operates in the reductive pentose phosphate cycle, is considerably slower (specific activity of 2–4 μmol $min^{-1}$ $mg^{-1}$) and has an incomplete specificity toward $CO_2$ in the presence of ambient concentrations of $O_2$ (Sage, 2002; Long *et al.*, 2006; Raines, 2006). The slowest enzyme of the 3-hydroxypropionate bicycle is also a carboxylating enzyme: propionyl-CoA carboxylase, which under ambient $CO_2$ concentration is expected to have a specific activity that is only somewhat higher than that of Rubisco. In fact, the pathway specific activity criterion suggests that the overall kinetics of the reductive pentose phosphate cycle, neglecting photorespiration, is similar to that of the 3-hydroxypropionate bicycle (~0.4 μmol (glyceraldehyde-3P) $min^{-1}$ $mg^{-1}$) (Bar-Even *et al.*, 2010). However, when photorespiration is considered, the 3-hydroxypropionate bicycle is expected to be 1.5 times faster than the reductive pentose phosphate cycle (Bar-Even *et al.*, 2010).

The carbon fixation cycles which use the carboxylating enzymes pyruvate synthase and 2-ketoglutarate synthase present a special challenge for kinetic estimation. The kinetics of the reductive reaction of these enzymes has not been well-characterized and the specific activities which were measured in the reductive direction are quite low. For example, pyruvate synthase from *Chlorobium tepidum* has a specific activity which is less than 0.1 μmol $min^{-1}$ $mg^{-1}$

(Yoon *et al.*, 1999). A similar enzyme from *Thermococcus litoralis* has a higher specific activity of ~2.3 μmol $min^{-1}$ $mg^{-1}$ but a very low affinity towards $CO_2$ ($K_M$=48 mM), making it extremely slow unless the $CO_2$ concentration is very high (Heider *et al.*, 1996). Finally, *Clostridium thermoaceticum* operates an enzyme with a higher affinity towards $CO_2$, $K_M$=2 mM, but a lower specific activity of 1.6 μmol $min^{-1}$ $mg^{-1}$ ($k_{cat}$=3.2 $s^{-1}$) (Furdui and Ragsdale, 2000).

Yet, some organisms that operate the reductive TCA cycle, using both pyruvate synthase and 2-ketoglutarate synthase, are known to have a very fast doubling time. For example, *Hydrogenobacter thermophilus* strain TK-6 can reach a doubling time of about 1 h when oxidizing hydrogen under aerobic conditions (Yamamoto *et al.*, 2006). It will be interesting to investigate how pyruvate and 2-ketoglutarate synthases have been evolved to facilitate such a fast growth.

### *Other factors that significantly affect the rate of carbon fixation*

An important factor that influences pathway kinetics but is not taken into account in the pathway specific activity criterion is the chemical motive force ($\Delta G_r$' for the reaction). The rate of a metabolic process is indirectly related to the chemical motive force: $\Delta G_r$' dictates what fraction of the enzymatic machinery catalyses the forward reaction (Beard and Qian, 2007; Qian *et al.*, 2003). High chemical motive force means that most of the enzymatic machinery is catalysing the forward reaction, resulting in a higher metabolic conversion rate. For example, $\Delta G_r$' of 6 kJ $mol^{-1}$ corresponds to ~90% of the enzymes catalysing the forward direction; hence, if the $k_{cat}$ values of the forward and backward reactions are similar, the reaction proceeds in ~80% of its maximal rate (assuming substrate saturation). On the other hand, low chemical motive force implies that a significant fraction of the enzymatic machinery catalyses the reverse direction, leading to lower net flux (Beard and Qian, 2007).

Since the reductive TCA cycle and the reductive acetyl-CoA pathway are expected to operate close to the thermodynamic edge, the chemical motive force supporting carbon fixation by these pathways is expected to be small. Hence, a large fraction of their enzymatic apparatus is expected to catalyse the backward reaction, increasing significantly the amount of total protein needed to support an adequate net carbon fixation flux. For example, the energetic challenge associated with CO-dehydrogenase results in a low chemical motive force for $CO_2$ reduction and further translates into a high protein requirement. Indeed, the CO-dehydrogenase-acetyl-CoA synthase complex represents a significant fraction of the soluble cell protein of acetogens (Drake *et al.*, 2006).

Another important parameter that affects pathway kinetics is the inorganic carbon species accepted by the carboxylating enzymes: $CO_2$ or bicarbonate. At a given pH, this preference is expected to have only a minor effect on pathway energetics since the inorganic carbon species are assumed to equilibrate relatively fast (Alberty, 2003). However, the pathway kinetics is highly dependent on this preference. The concentration of dissolved $CO_2$ depends on the partial pressure of $CO_2$ in the air and on the solubility of $CO_2$ in water, which in turn is a function of temperature and salinity, but not of pH (Hunter, 1998; Sander, 1999). By contrast, the bicarbonate concentration depends on the dissolved $CO_2$ concentration and on the pH (Hunter, 1998). At pH 7 and above, the concentration of the bicarbonate becomes progressively higher than that of dissolved $CO_2$. Therefore, at environments in which pH ⩾7, organisms that employ carboxylating enzymes that accept bicarbonate (rather than $CO_2$) are expected to work closer to substrate saturation (Berg *et al.*, 2007). As shown in Table 1, the 3-hydroxypropionate bicycle and the 3-hydroxypropionate-4-hydroxybutyrate cycle are the only carbon fixation pathways that utilize mainly bicarbonate.

### *Are there any other carbon fixation pathways out there?*

The existence of such a variety of carbon fixation pathways suggests that maybe some natural alternatives are yet to be revealed (Berg *et al.*, 2010*a*; Berg, 2011; Fuchs, 2011). Indeed, there are a few organisms that are known to grow autotrophically without any of the known carbon fixation pathways (Berg *et al.*, 2010*a*, *b*; Berg, 2011). Since most carbon fixation cycles have a similar underlying structure (Fig. 1), it is tempting to suggest that other solutions of the same general structure operate in nature. While the exact elucidation of such novel solutions will require extensive biochemical detective work, identifying the existence of key enzymes by genomic approaches might help in suggesting whether an organism is operating a unique variant of a carbon fixation pathway (Campbell and Cary, 2004; Chong *et al.*, 2007; Auernik *et al.*, 2008; Podar *et al.*, 2008).

In the following section we try to speculate what, as yet undiscovered, carbon fixation might look like. We stress that the pathways discussed below are ultimately no more than guesswork. However, such considerations might help obtain a deeper understanding of the known pathways as well as provide useful ideas for future genetic and biochemical research.

A relatively small metabolic deviation from the 3-hydroxypropionate bicycle would be the assimilation of glyoxylate not through citramalate (Fig. 1, column 'E') but using other metabolic alternatives. Figure 4A presents three existing pathways that assimilate glyoxylate into central metabolism by converting it either to glycerate or pyruvate. While some of these options are superior over the others in terms of energy efficiency, all three suffer from the same disadvantage with respect to the 3-hydroxypropionate bicycle: they release one $CO_2$ for every two glyoxylate molecules being assimilated. Therefore, a quarter of all the $CO_2$ molecules that were fixed are released again, decreasing the net efficiency of the carbon fixation process. For carbon fixation pathways that operate near the thermodynamic limit, this loss of $CO_2$ might be advantageous, making the

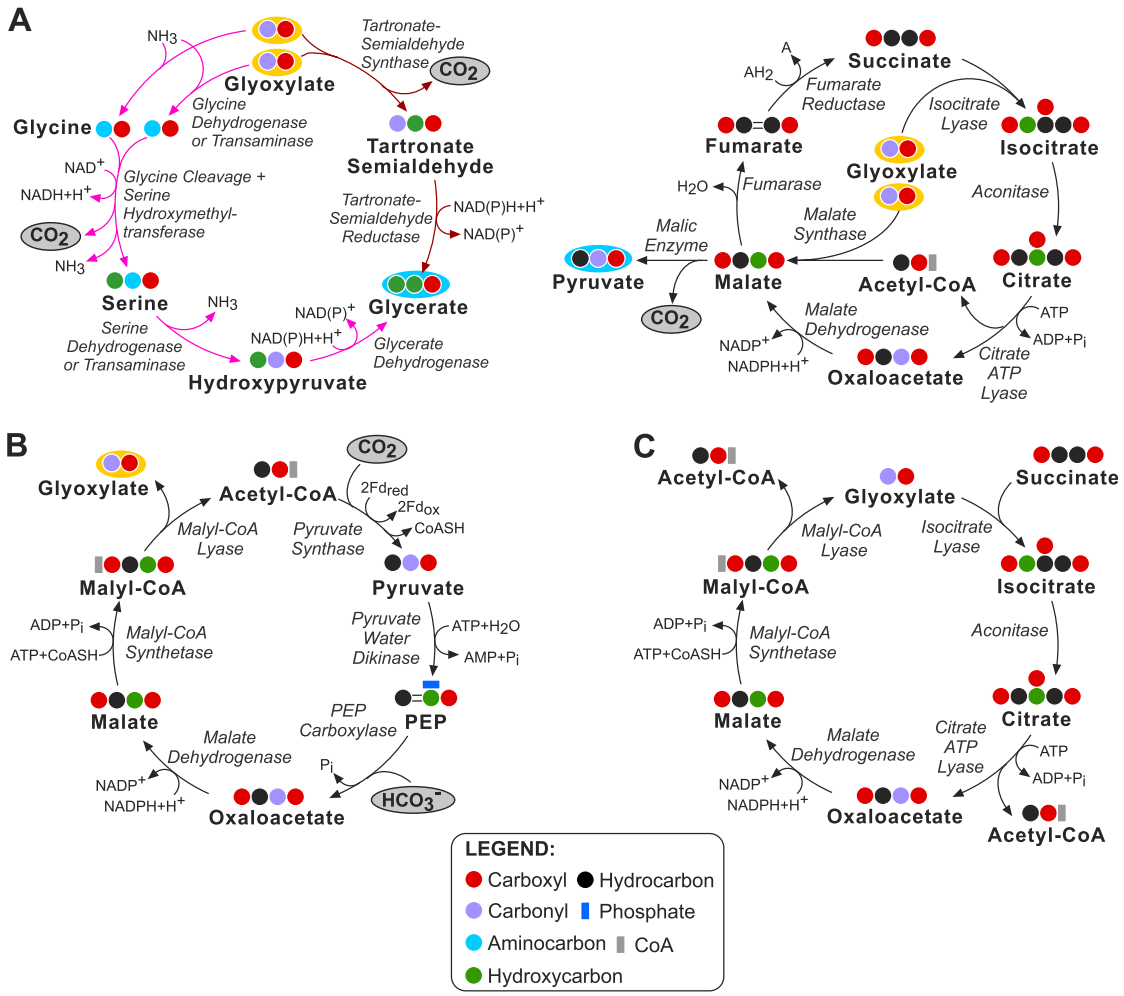


**Fig. 4.** Possible metabolic structures that might operate in yet undiscovered carbon fixation pathways.(A) Metabolic alternatives for the assimilation of glyoxylate into central metabolism. The magenta arrows of the left cycle correspond to the photorespiration pathway of plants (Kebeish *et al*., 2007; Bauwe *et al*., 2010), while the brown arrows correspond to the bacterial-like glycerate pathway (Eisenhut *et al*., 2006). The right cycle represents glyoxylate assimilation via the TCA cycle and the glyoxylate shunt. (B) A proposed carbon fixation cycle, which results from combining columns 'B' and 'F' of Fig. 1. (C) A proposed metabolic alternative of recycling succinyl-CoA into two acetyl-CoA molecules by a variant of the glyoxylate shunt, running in reverse.

energetics more favourable. However, since the 3-hydroxypropionate bicycle already involves the investment of seven ATP molecules, a further energetic investment is not required. It therefore seems that the bicycle provides a unique solution for glyoxylate assimilation without futile carbon release.

Figure 1 suggests that different combinations of downward metabolic arms (columns 'B' and 'D') and upward metabolic arms (columns 'A', 'C', and 'F'), which have not yet been found to operate in nature, might represent novel carbon fixation pathways. Of special interest is the combination of column 'B' with column 'F'. Since these columns contain some overlapping reactions, their combination 'shortcuts' many metabolic steps, giving rise to the short pathway shown in Fig. 4B. Glyoxylate, the cycle's product, can then be assimilated using the pathways discussed above (Fig. 4A). Since the energetics of this cycle is not as favourable as that of the 3-hydroxypropionate bicycle, the decarboxylation associated with glyoxylate assimilation might be beneficial in driving the cycle forward. Notably, this cycle was once suggested to operate in *Chloroflexus aurantiacus* B-3 (Ivanovsky, 1993), but this hypothesis has since been abandoned. It is an open challenge to the community to utilize state of the art biochemical and genomic capabilities to revisit this forgotten pathway.

Focusing on the upward metabolic arms of Fig. 1, another simple way to recycle succinyl-CoA into two acetyl-CoA molecules is by a variant of the glyoxylate shunt (Beeckmans, 2001) running in reverse (Fig. 4C). To energize the reverse metabolic flow of this cycle, two ATP molecules are hydrolysed by two enzymes that are not used by the normal glyoxylate shunt: ATP-citrate lyase which cleaves citrate to acetyl-CoA and oxaloacetate (see Supplementary Table S1 at *JXB* online) and malyl-CoA synthetase. The latter enzyme is somewhat exotic and was found to operate in only a small number of organisms (Hersh, 1973, 1974). However, this enzyme can be bypassed by another enzyme, succinyl-CoA-malate CoA transferase, which operates in

the 3-hydroxypropionate bicycle (see Supplementary Table S1 at *JXB* online) (Friedmann *et al.*, 2006). This enzyme directly transfers the CoA moiety of succinyl-CoA to malate, forming malyl-CoA (Friedmann *et al.*, 2006). The enzyme malyl-CoA lyase can then cleave malyl-CoA into acetyl-CoA and glyoxylate (Fig. 5; see Supplementary Table S1 at *JXB* online) (Hacking and Quayle, 1974, 1990; Herter *et al.*, 2002*a*; Meister *et al.*, 2005). Taken together, the reactions of this cycle are either abundant or can be readily evolved from existing enzymes. Moreover, none of the enzymes participating in the cycle represent special difficulties like oxygen sensitivity. Therefore, it is tempting to suggest that this metabolic alternative for succinyl-CoA cleavage might operate in some organisms.

Interestingly, the metabolic pathways used by methylotrophic organisms to assimilate reduced C1 compounds to central metabolism (Lidstrom, 2006; Seo *et al.*, 2007), can potentially be employed for autotrophic $CO_2$ assimilation. The ribulose monophosphate pathway and the xylulose 5-phosphate cycle assimilate free formaldehyde while the serine pathway assimilates formaldehyde while being attached to THF (5,10-methylene-THF) (Lidstrom, 2006; Seo *et al.*, 2007). Chemolithotrophic or phototrophic organisms can directly reduce $CO_2$ to formate, attach it to THF and reduce it to 5,10-methylene-THF, enabling carbon fixation through the serine pathway. Since 5,10-methylene-THF and its derivatives can be enzymatically or spontaneously cleaved to produce formaldehyde (Kallen and Jencks, 1966), the ribulose monophosphate pathway and the xylulose 5-phosphate cycle can also serve as effective $CO_2$ fixation pathways.

Deviating even further from the known pathways, the existence of a large repertoire of metabolic enzymes (~5000 known so far; Kanehisa and Goto, 2000) offers countless combinations that can potentially sustain carbon fixation. In a previous study, computational tools were used systematically to locate and analyse pathways that are composed of naturally occurring enzymes and capable of carbon fixation (Bar-Even *et al.*, 2010). Numerous possible thermodynamically feasible and kinetically viable pathways have been identified. In particular, a new family of carbon fixation pathways is suggested that makes use of the most effective carboxylating enzyme, PEP carboxylase and of the metabolic module used in the efficient $C_4$ plants (Bar-Even *et al.*, 2010). While most of these alternatives probably exist only *in silico*, it is possible that some actually operate *in vivo*, waiting to be discovered.

### *Concluding remarks*

The diversity of metabolic alternatives for carbon fixation, each with its own unique characteristics, is a treasure for metabolic engineering, as it enables fitting an organism with the most appropriate pathway (Boyle and Morgan, 2011). Expressing a foreign carbon fixation within a host might serve many goals. For example, switching the carbon fixation pathway of a plant or alga can potentially increase its productivity under specific desirable conditions. Also, adopting a biotechnologically important heterotrophic microbe, such as *E. coli* or *S. cerevisiae*, to an autotrophic mode of growth is a promising venue for the production of various commodities without the need of costly feedstock which compete with human and animal consumption (DOE, 2011; Hawkins *et al.*, 2011). In all of these cases, it is important to pick the right carbon fixation pathway for the host. In choosing a suitable pathway one should consider, for example, the following aspects: (i) Does the oxygen tolerance of the pathway suit the intended cultivation conditions? (ii) Does the ATP requirement of the pathway fit the expected cellular energy influx? (iii) Does the expected kinetics of the pathway coincide with the desirable growth rate? (iv) Does the structure of the pathway integrate well with the endogenous metabolic network? (v) Do the optimal temperatures of the enzymes participating in the chosen pathway fit the intended cultivation temperature?

Regardless of the reason for which one is interested in carbon fixation, whether it is purely scientific or biotechnological, many more interesting discoveries can still be expected in the years to come. The repertoire of carbon fixation pathways currently known is expected to grow as more organisms are cultivated, sequenced, and biochemically analysed, so it may well be possible to build a more comprehensive picture of the design principles of carbon fixation pathways.

## Supplementary data

Supplementary data can be found at *JXB* online.

**Supplementary Table S1**. Enzymes of the acetyl-CoA-succinyl-CoA carbon fixation cycles (as shown in Fig. 1).

**Supplementary Table S2.** Enzymes of the C1 carbon fixation pathways (as shown in Fig. 2).

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## References

**Alberty RA.** 2003. *Thermodynamics of biochemical reactions:* Wiley-Interscience.

**Andreesen JR.** 2004. Glycine reductase mechanism. *Current Opinion in Chemical Biology* **8,** 454–461.

**Auernik KS, Maezato Y, Blum PH, Kelly RM.** 2008. The genome sequence of the metal-mobilizing, extremely thermoacidophilic archaeon

*Metallosphaera sedula* provides insights into bioleaching-associated metabolism. *Applied Environmental Microbiology* **74,** 682–692.

**Bar-Even A, Noor E, Flamholz A, Buescher JM, Milo R.** 2011*a*. Hydrophobicity and charge shape cellular metabolite concentrations. *PLoS Computational Biology* 7, e1002166.

**Bar-Even A, Noor E, Lewis NE, Milo R.** 2010. Design and analysis of synthetic carbon fixation pathways. *Proceedings of the National Academy of Sciences, USA* **107,** 8889–8894.

**Bar-Even A, Noor E, Savir Y, Liebermeister W, Davidi D, Tawfik DS, Milo R.** 2011*b*. The moderately efficient enzyme: evolutionary and physicochemical trends shaping enzyme parameters. *Biochemistry* **50,** 4402–4410.

**Bassham JA, Benson AA, Calvin M.** 1950. The path of carbon in photosynthesis. *Journal of Biological Chemistry* **185,** 781–787.

**Bauwe H, Hagemann M, Fernie AR.** 2010. Photorespiration: players, partners and origin. *Trends in Plant Science* **15,** 330–336.

**Beard DA, Qian H.** 2007. Relationship between thermodynamic driving force and one-way fluxes in reversible processes. *PLoS One* **2,** e144.

**Beeckmans S.** 2001. *Glyoxylate cycle. Encyclopedia of Life Sciences*. Chichester, UK: John Wiley & Sons Ltd.

**Beh M, Strauss G, Huber H, Stetter K-O, Fuchs G.** 1993. Enzymes of the reductive citric acid cycle in the autotrophic eubacterium *Aquifex pyrophilus* and in the archaebacterium *Thermoproteus neutrophilus*. *Archives of Microbiology* **160,** 306–311.

**Bennett BD, Kimball EH, Gao M, Osterhout R, Van Dien SJ, Rabinowitz JD.** 2009. Absolute metabolite concentrations and implied enzyme active site occupancy in. *Escherichia coli*. *Nature Chemical Biology* **5,** 593–599.

**Berg IA.** 2011. Ecological aspects of the distribution of different autotrophic $CO_2$ fixation pathways. *Applied Environmental Microbiology* **77,** 1925–1936.

**Berg IA, Kockelkorn D, Buckel W, Fuchs G.** 2007. A 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway in Archaea. *Science* **318,** 1782–1786.

**Berg IA, Kockelkorn D, Ramos-Vera WH, Say RF, Zarzycki J, Hugler M, Alber BE, Fuchs G.** 2010*a*. Autotrophic carbon fixation in archaea. *Nature Reviews Microbiology* **8,** 447–460.

**Berg IA, Ramos-Vera WH, Petri A, Huber H, Fuchs G.** 2010*b*. Study of the distribution of autotrophic $CO_2$ fixation cycles in Crenarchaeota. *Microbiology* **156,** 256–269.

**Biegel E, Muller V.** 2010. Bacterial $Na^+$-translocating ferredoxin:NAD+oxidoreductase. *Proceedings of the National Academy of Sciences, USA* **107,** 18138–18142.

**Blankenship RE.** 2002. *Molecular mechanisms of photosynthesis*: Oxford: Wiley-Blackwell.

**Boyle NR, Morgan JA.** 2011. Computation of metabolic fluxes and efficiencies for biological carbon dioxide fixation. *Metabolic Engineering* **13,** 150–158.

**Brune DC.** 1989. Sulfur oxidation by phototrophic bacteria. *Biochimica et Biophysica Acta* **975,** 189–221.

**Buchanan BB, Arnon DI.** 1990. A reverse KREBS cycle in photosynthesis: consensus at last. *Photosynthesis Research* **24,** 47–53.

**Campbell BJ, Cary SC.** 2004. Abundance of reverse tricarboxylic acid cycle genes in free-living microorganisms at deep-sea hydrothermal vents. *Applied Environmental Microbiology* **70,** 6282–6289.

**Chong PK, Burja AM, Radianingtyas H, Fazeli A, Wright PC.** 2007. Proteome and transcriptional analysis of ethanol-grown *Sulfolobus solfataricus* P2 reveals ADH2, a potential alcohol dehydrogenase. *Journal of Proteome Research* **6,** 3985–3994.

**Collins MD, Lawson PA, Willems A, Cordoba JJ, Fernandez-Garayzabal J, Garcia P, Cai J, Hippe H, Farrow JA.** 1994. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *International Journal of Systematic Bacteriology* **44,** 812–826.

**Deppenmeier U, Müller V.** 2008. Life close to the thermodynamic limit: how methanogenic archaea conserve energy. In: Schafer G, Penefsky H, eds. *Bioenergetics: energy conservation and conversion*. Springer.

**DiMarco AA, Bobik TA, Wolfe RS.** 1990. Unusual coenzymes of methanogenesis. *Annu Rev Biochem* **59,** 355–394.

**DiSpirito AA, Tuovinen OH.** 1982. Uranous ion oxidation and carbon dioxide fixation by *Thiobacillus ferrooxidans*. *Archives of Microbiology* **133,** 28–32.

**Dobrinski KP.** 2009. *Thiomicrospira crunogena*: a chemoautotroph with a carbon concentrating mechanism. University of South Florida.

**DOE.** 2011. *ARPA-E electrofuels project summary*. http://arpa-e.energy.gov/LinkClick.aspx?fileticket=TRv8LDEgnhw%3D&tabid=82.

**Drake HL.** 1994. Acetogenesis, acetogenic bacteria, and the acetyl-CoA 'Wood–Ljungdahl' pathway: past and current perspectives. In: Drake HL, ed. *Acetogenesis*. New York: Chapman and Hall, 3–60.

**Drake HL, Gossner AS, Daniel SL.** 2008. Old acetogens, new light. *Annals of the New York Academy of Sciences* **1125,** 100–128.

**Drake HL, Kirsten K, Matthies C.** 2006. Acetogenic prokaryotes. In: *The Prokaryotes*. New York: Springer, 354–420.

**Durre P, Andreesen JR.** 1982. Pathway of carbon dioxide reduction to acetate without a net energy requirement in *Clostridium purinolyticum*. *FEMS Microbiology Letters* **15,** 51–56.

**Durre P, Spahr R, Andreesen JR.** 1983. Glycine fermentation via a glycine reductase in *Peptococcus glycinophilus* and *Peptococcus magnus*. *Archives of Microbiology* **134,** 127–135.

**Eisenhut M, Kahlon S, Hasse D, Ewald R, Lieman-Hurwitz J, Ogawa T, Ruth W, Bauwe H, Kaplan A, Hagemann M.** 2006. The plant-like C2 glycolate cycle and the bacterial-like glycerate pathway cooperate in phosphoglycolate metabolism in cyanobacteria. *Plant Physiology* **142,** 333–342.

**Elbehti A, Brasseur G, Lemesle-Meunier D.** 2000. First evidence for existence of an uphill electron transfer through the bc(1) and NADH-Q oxidoreductase complexes of the acidophilic obligate chemolithotrophic ferrous ion-oxidizing bacterium *Thiobacillus ferrooxidans*. *Journal of Bacteriology* **182,** 3602–3606.

**Escalante-Semerena JC, Leigh JA, Rinehart KL, Wolfe RS.** 1984*a*. Formaldehyde activation factor, tetrahydromethanopterin,

a coenzyme of methanogenesis. *Proceedings of the National Academy of Sciences, USA* **81,** 1976–1980.

**Escalante-Semerena JC, Rinehart Jr KL, Wolfe RS.** 1984*b*. Tetrahydromethanopterin, a carbon carrier in methanogenesis. *Journal of Biological Chemistry* **259,** 9447–9455.

**Estelmann S, Hugler M, Eisenreich W, Werner K, Berg IA, Ramos-Vera WH, Say RF, Kockelkorn D, Gad'on N, Fuchs G.** 2011. Labeling and enzyme studies of the central carbon metabolism in *Metallosphaera sedula*. *Journal of Bacteriology* **193,** 1191–1200.

**Evans MC, Buchanan BB, Arnon DI.** 1966. A new ferredoxin-dependent carbon reduction cycle in a photosynthetic bacterium. *Proceedings of the National Academy of Sciences, USA* **55,** 928–934.

**Fell DA.** 1992. Metabolic control analysis: a survey of its theoretical and experimental development. *Biochemical Journal* **286,** 313–330.

**Flamholz A, Noor E, Bar-Even A, Milo R.** 2011. eQuilibrator–the biochemical thermodynamics calculator. *Nucleic Acids Res.*

**Fonknechten N, Chaussonnerie S, Tricot S, *et al*.** 2010. *Clostridium sticklandii*, a specialist in amino acid degradation:revisiting its metabolism through its genome sequence. *BMC Genomics* **11,** 555.

**Fontaine FE, Peterson WH, McCoy E, Johnson MJ, Ritter GJ.** 1942. A new type of glucose fermentation by *Clostridium thermoaceticum*. *Journal of Bacteriology* **43,** 701–715.

**Friedmann S, Steindorf A, Alber BE, Fuchs G.** 2006. Properties of succinyl-coenzyme A:L-malate coenzyme A transferase and its role in the autotrophic 3-hydroxypropionate cycle of *Chloroflexus aurantiacus*. *Journal of Bacteriology* **188,** 2646–2655.

**Fuchs G.** 1985. $CO_2$ fixation in acetogenic bacteria: variations on a theme. *FEMS Microbiology Letters* **39,** 181–213.

**Fuchs G.** 2011. Alternative pathways of carbon dioxide fixation: insights into the early evolution of life. *Annual Review of Microbiology* **65,** 631–658.

**Furdui C, Ragsdale SW.** 2000. The role of pyruvate ferredoxin oxidoreductase in pyruvate synthesis during autotrophic growth by the Wood–Ljungdahl pathway. *Journal of Biological Chemistry* **275,** 28494–28499.

**Giordano M, Beardall J, Raven JA.** 2005. $CO_2$ concentrating mechanisms in algae: mechanisms, environmental modulation, and evolution. *Annual Review of Plant Biology* **56,** 99–131.

**Hacking AJ, Quayle JR.** 1974. Purification and properties of malyl-coenzyme A lyase from *Pseudomonas* AM1. *Biochemical Journal* **139,** 399–405.

**Hacking AJ, Quayle JR.** 1990. Malyl-CoA lyase from *Methylobacterium extorquens* AM1. *Methods in Enzymology* **188,** 379–386.

**Hauska G, Schoedl T, Remigy H, Tsiotis G.** 2001. The reaction center of green sulfur bacteria(1). *Biochimica et Biophysica Acta* **1507,** 260–277.

**Hawkins AS, Han Y, Lian H, Loder AJ, Menon AL, Iwuchukwu IJ, Keller M, Leuko TT, Adams MWW, Kelly RM.** 2011. Extremely thermophilic routes to microbial electrofuels. *ACS Catalysis* **1,** 1043–1050.

**Heider J, Mai X, Adams MW.** 1996. Characterization of 2-ketoisovalerate ferredoxin oxidoreductase, a new and reversible coenzyme A-dependent enzyme involved in peptide fermentation by hyperthermophilic archaea. *Journal of Bacteriology* **178,** 780–787.

**Heinhorst S, Williams EB, Cai F, Murin CD, Shively JM, Cannon GC.** 2006. Characterization of the carboxysomal carbonic anhydrase CsoSCA from *Halothiobacillus neapolitanus*. *Journal of Bacteriology* **188,** 8087–8094.

**Henry CS, Jankowski MD, Broadbelt LJ, Hatzimanikatis V.** 2006. Genome-scale thermodynamic analysis of *Escherichia coli* metabolism. *Biophysical Journal* **90,** 1453–1461.

**Hersh LB.** 1973. Malate adenosine triphosphate lyase. Separation of the reaction into a malate thiokinase and malyl coenzyme A lyase. *Journal of Biological Chemistry* **248,** 7295–7303.

**Hersh LB.** 1974. Malate thiokinase. The reaction mechanism as determined by initial rate studies. *Journal of Biological Chemistry* **249,** 6264–6271.

**Herter S, Busch A, Fuchs G.** 2002*a*. L-Malyl-coenzyme A lyase/beta-methylmalyl-coenzyme A lyase from *Chloroflexus aurantiacus*, a bifunctional enzyme involved in autotrophic CO(2) fixation. *Journal of Bacteriology* **184,** 5999–6006.

**Herter S, Fuchs G, Bacher A, Eisenreich W.** 2002*b*. A bicyclic autotrophic CO2 fixation pathway in *Chloroflexus aurantiacus*. *Journal of Biological Chemistry* **277,** 20277–20283.

**Huber H, Gallenberger M, Jahn U, Eylert E, Berg IA, Kockelkorn D, Eisenreich W, Fuchs G.** 2008. A dicarboxylate/4-hydroxybutyrate autotrophic carbon assimilation cycle in the hyperthermophilic Archaeum. *Ignicoccus hospitalis*. *Proceedings of the National Academy of Sciences, USA* **105,** 7851–7856.

**Hugler M, Huber H, Molyneaux SJ, Vetriani C, Sievert SM.** 2007. Autotrophic $CO_2$ fixation via the reductive tricarboxylic acid cycle in different lineages within the phylum Aquificae: evidence for two ways of citrate cleavage. *Environmental Microbiology* **9,** 81–92.

**Hugler M, Sievert SM.** 2011. Beyond the Calvin cycle: autotrophic carbon fixation in the ocean. *Annual Review of Marine Science* **3,** 261–289.

**Hunter AH.** 1998. *Acid–base chemistry of aquatic systems*. Dunedin, New Zealand: Department of Chemistry, University of Otago.

**Imkamp I, Muller M.** 2007. *Acetogenic bacteria*. Encyclopedia of life sciences. Chichester, UK: John Wiley & Sons.

**Imlay JA.** 2006. Iron–sulphur clusters and the problem with oxygen. *Molecular Microbiology* **59,** 1073–1082.

**Ivanovsky RN.** 1993. A pathway of the autotrophic $CO_2$ fixation in *Chloroflexus aurantiacus*. *Archives of Microbiology* **159,** 257–264.

**Janave MT, Ramaswamy NK, Nair PM.** 1993. Purification and characterization of glyoxylate synthetase from greening potato-tuber chloroplasts. *European Journal of Biochemistry* **214,** 889–896.

**Janave MT, Ramaswamy NK, Nair PM.** 1999. Studies on determination of active site amino acid residues in glyoxylate synthetase from potato tuber chloroplasts. *Plant Physiology and Biochemistry* **37,** 121–129.

**Jankowski MD, Henry CS, Broadbelt LJ, Hatzimanikatis V.** 2008. Group contribution method for thermodynamic analysis of complex metabolic networks. *Biophysical Journal* **95,** 1487–1499.

**Joshi GS, Romagnoli S, Verberkmoes NC, Hettich RL, Pelletier D, Tabita FR.** 2009. Differential accumulation of form I RubisCO in *Rhodopseudomonas palustris* CGA010 under photoheterotrophic growth conditions with reduced carbon sources. *Journal of Bacteriology* **191,** 4243–4250.

**Kallen RG, Jencks WP.** 1966. The mechanism of the condensation of formaldehyde with tetrahydrofolic acid. *Journal of Biological Chemistry* **241,** 5851–5863.

**Kanehisa M, Goto S.** 2000. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Research* **28,** 27–30.

**Kaplan A, Reinhold L.** 1999. $CO_2$ concentrating mechanisms in photosynthetic microorganisms. *Annual Review of Plant Physiology and Plant Molecular Biology* **50,** 539–570.

**Kebeish R, Niessen M, Thiruveedhi K, Bari R, Hirsch HJ, Rosenkranz R, Stabler N, Schonfeld B, Kreuzaler F, Peterhansel C.** 2007. Chloroplastic photorespiratory bypass increases photosynthesis and biomass production in Arabidopsis thaliana. *Nature Biotechnology* **25,** 593–599.

**Keltjens JT, Huberts MJ, Laarhoven WH, Vogels GD.** 1983. Structural elements of methanopterin, a novel pterin present in *Methanobacterium thermoautotrophicum*. *European Journal of Biochemistry* **130,** 537–544.

**Kerscher L, Oesterhelt D.** 1981. The catalytic mechanism of 2-oxoacid:ferredoxin oxidoreductases from *Halobacterium halobium*. One-electron transfer at two distinct steps of the catalytic cycle. *European Journal of Biochemistry* **116,** 595–600.

**Kikuchi G.** 1973. The glycine cleavage system: composition, reaction mechanism, and physiological significance. *Molecular and Cellular Biochemistry* **1,** 169–187.

**Klamt S, Grammel H, Straube R, Ghosh R, Gilles ED.** 2008. Modeling the electron transport chain of purple non-sulfur bacteria. *Molecular Systems Biology* **4,** 156.

**Kruger NJ, Ratcliffe RG.** 2009. Insights into plant metabolic networks from steady-state metabolic flux analysis. *Biochimie* **91,** 697–702.

**Libourel IG, Shachar-Hill Y.** 2008. Metabolic flux analysis in plants: from intelligent design to rational engineering. *Annual Review of Plant Biology* **59,** 625–650.

**Lidstrom ME.** 2006. Aerobic methylotrophic prokaryotes. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E, eds. *The prokaryotes*. New York: Springer, 618–634.

**Liegel JM.** 1985. *The equilibrium constant for the glycine synthase reaction*. University of Texas.

**Ljungdahl LG.** 1986. The autotrophic pathway of acetate synthesis in acetogenic bacteria. *Annual Review of Microbiology* **40,** 415–450.

**Ljungdahl LG.** 2009. A life with acetogens, thermophiles, and cellulolytic anaerobes. *Annual Review of Microbiology* **63,** 1–25.

**Long SP, Zhu XG, Naidu SL, Ort DR.** 2006. Can improvement in photosynthesis increase crop yields? *Plant, Cell and Environment* **29,** 315–330.

**Maden BE.** 2000. Tetrahydrofolate and tetrahydromethanopterin compared: functionally distinct carriers in C1 metabolism. *Biochemical Journal* **350,** 609–629.

**Manske AK, Glaeser J, Kuypers MM, Overmann J.** 2005. Physiology and phylogeny of green sulfur bacteria forming a monospecific phototrophic assemblage at a depth of 100 meters in the Black Sea. *Applied Environmental Microbiology* **71,** 8049–8060.

**Martin WF.** 2011. Hydrogen, metals, bifurcating electrons, and proton gradients: the early evolution of biological energy conservation. *FEBS Letters* doi:10.1016/j.febslet.2011.09.031.

**Martins BM, Dobbek H, Cinkaya I, Buckel W, Messerschmidt A.** 2004. Crystal structure of 4-hydroxybutyryl-CoA dehydratase: radical catalysis involving a [4Fe-4S] cluster and flavin. *Proceedings of the National Academy of Sciences, USA* **101,** 15645–15649.

**McCollom TM, Amend JP.** 2005. A thermodynamic assessment of energy requirements for biomass synthesis by chemolithoautotrophic micro-organisms in oxic and anoxic environments. *Geobiology* **3,** 135–144.

**Meister M, Saum S, Alber BE, Fuchs G.** 2005. L-malyl-coenzyme A/beta-methylmalyl-coenzyme A lyase is involved in acetate assimilation of the isocitrate lyase-negative bacterium *Rhodobacter capsulatus*. *Journal of Bacteriology* **187,** 1415–1425.

**Minic Z, Thongbam PD.** 2011. The biological deep sea hydrothermal vent as a model to study carbon dioxide capturing enzymes. *Marine Drugs* **9,** 719–738.

**Morgan JA, Rhodes D.** 2002. Mathematical modeling of plant metabolic pathways. *Metabolic Engineering* **4,** 80–89.

**Naser U, Pierik AJ, Scott R, Cinkaya I, Buckel W, Golding BT.** 2005. Synthesis of (13)C-labeled gamma-hydroxybutyrates for EPR studies with 4-hydroxybutyryl-CoA dehydratase. *Bioorganic Chemistry* **33,** 53–66.

**Overmann J, Cypionka H, Pfennig N.** 1992. An extremely low-light-adapted phototrophic sulfur bacterium from the Black Sea. *Limnology and oceanography* **37,** 150–155.

**Pasternack LB, Laude Jr DA, Appling DR.** 1992. $^{13}C$ NMR detection of folate-mediated serine and glycine synthesis *in vivo* in *Saccharomyces cerevisiae*. *Biochemistry* **31,** 8713–8719.

**Peterhansel C, Niessen M, Kebeish RM.** 2008. Metabolic engineering towards the enhancement of photosynthesis. *Photochemistry and Photobiology* **84,** 1317–1323.

**Pieulle L, Magro V, Hatchikian EC.** 1997. Isolation and analysis of the gene encoding the pyruvate-ferredoxin oxidoreductase of *Desulfovibrio africanus*, production of the recombinant enzyme in *Escherichia coli*, and effect of carboxy-terminal deletions on its stability. *Journal of Bacteriology* **179,** 5684–5692.

**Podar M, Anderson I, Makarova KS, *et al*.** 2008. A genomic analysis of the archaeal system *Ignicoccus hospitalis–Nanoarchaeum equitans*. *Genome Biology* **9,** R158.

**Poolman MG, Fell DA, Thomas S.** 2000. Modelling photosynthesis and its control. *Journal of Experimental Botany* **51,** 319–328.

**Pratscher J, Dumont MG, Conrad R.** 2011. Ammonia oxidation coupled to $CO_2$ fixation by archaea and bacteria in an agricultural soil.

*Proceedings of the National Academy of Sciences, USA* **108,** 4170–4175.

**Price GD, Badger MR, Woodger FJ, Long BM.** 2008. Advances in understanding the cyanobacterial $CO_2$-concentrating-mechanism (CCM): functional components, $C_i$ transporters, diversity, genetic regulation, and prospects for engineering into plants. *Journal of Experimental Botany* **59,** 1441–1461.

**Qian H, Beard DA, Liang SD.** 2003. Stoichiometric network theory for nonequilibrium biochemical systems. *European Journal of Biochemistry* **270,** 415–421.

**Ragsdale SW.** 2003. Pyruvate ferredoxin oxidoreductase and its radical intermediate. *Chemical Reviews* **103,** 2333–2346.

**Ragsdale SW.** 2008. Enzymology of the Wood–Ljungdahl pathway of acetogenesis. *Annals of the New York Academy of Science* **1125,** 129–136.

**Ragsdale SW, Pierce E.** 2008. Acetogenesis and the Wood–Ljungdahl pathway of CO(2) fixation. *Biochimica et Biophysica Acta* **1784,** 1873–1898.

**Raines CA.** 2003. The Calvin cycle revisited. *Photosynthesis Research* **75,** 1–10.

**Raines CA.** 2006. Transgenic approaches to manipulate the environmental responses of the C3 carbon fixation cycle. *Plant, Cell and Environment* **29,** 331–339.

**Ramaswamy NK, Behere AG, Nair PM.** 1976. A novel pathway for the synthesis of solanidine in the isolated chloroplast from greening potatoes. *European Journal of Biochemistry* **67,** 275–282.

**Ramaswamy NK, Nair PM.** 1984. Evidence for the operation of a C1-pathway for the fixation of $CO_2$ in isolated intact chloroplasts from green potato tubers. *Plant Science Letters* **34,** 261–267.

**Ramaswamy NK, Sangeeta GJ.** 1983. Glyoxylate synthetase isolated from green potato tuber chloroplasts catalysing the conversion of formate to glyoxylate. *Plant Science Letters* **32,** 213–220.

**Ramos-Vera WH, Weiss M, Strittmatter E, Kockelkorn D, Fuchs G.** 2011. Identification of missing genes and enzymes for autotrophic carbon fixation in crenarchaeota. *Journal of Bacteriology* **193,** 1201–1211.

**Ringe D, Petsko GA.** 2008. Biochemistry. How enzymes work. *Science* **320,** 1428–1429.

**Sage RF.** 2002. Variation in the k(cat) of Rubisco in C(3) and C(4) plants and some implications for photosynthetic performance at high and low temperature. *Journal of Experimental Botany* **53,** 609–620.

**Sander R.** 1999. Compilation of Henry's Law constants for inorganic and organic species of potential importance in environmental chemistry (Version 3). http://www.henrys-law.org.

**Sato T, Atomi H.** 2010. *Microbial inorganic carbon fixation*. Encyclopedia of Life Sciences. Chichester, UK: John Wiley and Sons Ltd.

**Schiefer-Ullrich H, Wagner R, Durre P, Andreesen JR.** 1984. Comparative studies on physiology and taxonomy of obligately purinolytic clostridia. *Archives of Microbiology* **138,** 345–353.

**Schneeberger A, Frings J, Schink B.** 1999. Net synthesis of acetate from $CO_2$ by *Eubacterium acidaminophilum* through the glycine reductase pathway. *FEMS Microbiology Letters* **177,** 1–6.

**Schwender J, Ohlrogge J, Shachar-Hill Y.** 2004. Understanding flux in plant metabolic networks. *Current Opinion in Plant Biology* **7,** 309–317.

**Scott R, Naser U, Friedrich P, Selmer T, Buckel W, Golding BT.** 2004. Stereochemistry of hydrogen removal from the 'unactivated' C-3 position of 4-hydroxybutyryl-CoA catalysed by 4-hydroxybutyryl-CoA dehydratase. *Chemical Communications (Camb)* 1210–1211.

**Seo JG, Park SW, Park H, Kim SY, Ro YT, Kim E, Cho JW, Kim YM.** 2007. Cloning, characterization and expression of a gene encoding dihydroxyacetone synthase in *Mycobacterium* sp. strain JC1 DSM 3803. *Microbiology* **153,** 4174–4182.

**Shiba H, Kawasumi T, Igarashi Y, Kodama T, Minoda Y.** 1985. The $CO_2$ assimilation via the reductive tricarboxylic acid cycle in an obligately autotrophic, aerobic hydrogen-oxidizing bacterium, *Hydrogenobacter thermophilus*. *Archives of Microbiology* **141,** 198–203.

**Stitt M.** 2004. Metabolic regulation of photosynthesis. In: Baker NR, ed. *Photosynthesis and the Environment*, Vol. 5. Dordrecht: Kluwer, 151–190.

**Stitt M, Schulze D.** 1994. Does Rubisco control the rate of photosynthesis and plant growth? An exercise in molecular ecophysiology. *Plant, Cell and Environment* **17,** 465–487.

**Stouthamer AH, de Boer AP, van der Oost J, van Spanning RJ.** 1997. Emerging principles of inorganic nitrogen metabolism in *Paracoccus denitrificans* and related bacteria. *Antonie Van Leeuwenhoek* **71,** 33–41.

**Tichi MA, Tabita FR.** 2000. Maintenance and control of redox poise in *Rhodobacter capsulatus* strains deficient in the Calvin–Benson–Bassham pathway. *Archives of Microbiology* **174,** 322–333.

**Tishkov VI, Popov VO.** 2006. Protein engineering of formate dehydrogenase. *Biomolecular Engineering* **23,** 89–110.

**Tran QH, Unden G.** 1998. Changes in the proton potential and the cellular energetics of *Escherichia coli* during growth by aerobic and anaerobic respiration or by fermentation. *European Journal of Biochemistry* **251,** 538–543.

**Vogels GD, Van der Drift C.** 1976. Degradation of purines and pyrimidines by microorganisms. *Bacteriological Reviews* **40,** 403–468.

**Waber LJ, Wood HG.** 1979. Mechanism of acetate synthesis from $CO_2$ by *Clostridium acidiurici*. *Journal of Bacteriology* **140,** 468–478.

**Wackerhage H, Hoffmann U, Essfeld D, Leyk D, Mueller K, Zange J.** 1998. Recovery of free ADP, Pi, and free energy of ATP hydrolysis in human skeletal muscle. *Journal of Applied Physiology* **85,** 2140–2145.

**Walker CB, de la Torre JR, Klotz MG, *et al*.** 2010. *Nitrosopumilus maritimus* genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. *Proeedings of the National Academy of Sciences, USA* **107,** 8818–8823.

**Wang X, Falcone DL, Tabita FR.** 1993. Reductive pentose phosphate-independent $CO_2$ fixation in *Rhodobacter sphaeroides* and evidence that ribulose bisphosphate carboxylase/oxygenase activity

serves to maintain the redox balance of the cell. *Journal of Bacteriology* **175,** 3372–3379.

**Weber AL.** 2002. Chemical constraints governing the origin of metabolism: the thermodynamic landscape of carbon group transformations under mild aqueous conditions. *Origins of Life and Evolution of the Biosphere* **32,** 333–357.

**Wright BE, Butler MH, Albe KR.** 1992. Systems analysis of the tricarboxylic acid cycle in *Dictyostelium discoideum*. I. The basis for model construction. *Journal of Biological Chemistry* **267,** 3101–3105.

**Yamamoto M, Arai H, Ishii M, Igarashi Y.** 2003. Characterization of two different 2-oxoglutarate:ferredoxin oxidoreductases from *Hydrogenobacter thermophilus* TK-6. *Biochemical and Biophysical Research Communications* **312,** 1297–1302.

**Yamamoto M, Arai H, Ishii M, Igarashi Y.** 2006. Role of two 2-oxoglutarate:ferredoxin oxidoreductases in *Hydrogenobacter thermophilus* under aerobic and anaerobic conditions. *FEMS Microbiological Letters* **263,** 189–193.

**Yeates TO, Kerfeld CA, Heinhorst S, Cannon GC, Shively JM.** 2008. Protein-based organelles in bacteria: carboxysomes and related microcompartments. *Nature Reviews Microbiology* **6,** 681–691.

**Yoon KS, Hille R, Hemann C, Tabita FR.** 1999. Rubredoxin from the green sulfur bacterium *Chlorobium tepidum* functions as an electron acceptor for pyruvate ferredoxin oxidoreductase. *Journal of Biological Chemistry* **274,** 29772–29778.

**Young JD, Shastri AA, Stephanopoulos G, Morgan JA.** 2011. Mapping photoautotrophic metabolism with isotopically nonstationary (13)C flux analysis. *Metabolic Engineering* **13,** 656–665.

**Zhang Q, Wiskich JT.** 1995. Activation of glycine decarboxylase in pea leaf mitochondria by ATP. *Archives of Biochemistry and Biophysics* **320,** 250–256.