# Alternative Pathways of Carbon Dioxide Fixation: Insights into the Early Evolution of Life?

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Annu. Rev. Microbiol. 2011. 65:631–58

First published online as a Review in Advance on July 6, 2011

The *Annual Review of Microbiology* is online at micro.annualreviews.org

This article's doi: 10.1146/annurev-micro-090110-102801



0066-4227/11/1013-0631$20.00

## Keywords

autotrophy, chemical evolution, carbon metabolism, carboxylases, origin of life, prebiotic chemistry

## Abstract

The fixation of inorganic carbon into organic material (autotrophy) is a prerequisite for life and sets the starting point of biological evolution. In the extant biosphere the reductive pentose phosphate (Calvin-Benson) cycle is the predominant mechanism by which many prokaryotes and all plants fix $CO_2$ into biomass. However, the fact that five alternative autotrophic pathways exist in prokaryotes is often neglected. This bias may lead to serious misjudgments in models of the global carbon cycle, in hypotheses on the evolution of metabolism, and in interpretations of geological records. Here, I review these alternative pathways that differ fundamentally from the Calvin-Benson cycle. Revealingly, these five alternative pathways pivot on acetyl-coenzyme A, the turntable of metabolism, demanding a gluconeogenic pathway starting from acetyl-coenzyme A and $CO_2$. It appears that the formation of an activated acetic acid from inorganic carbon represents the initial step toward metabolism. Consequently, biosyntheses likely started from activated acetic acid and gluconeogenesis preceded glycolysis.

## Contents



## INTRODUCTION

"Who among us would not be happy to lift the veil behind which is hidden the future; to gaze at the coming developments of our science and at the secrets of its development in the centuries to come?" With these words, David Hilbert put forth in 1900 a most influential list of unsolved mathematical problems, convinced "we must know—we will know!" Some of these problems were solved within a short time, a few are now taken to be unsuitably open-ended to be solved,

and some continue even to this day to remain a challenge. Before, Emil du Bois-Reymond put forth in 1880 seven biological *Welträtsel* (world riddles), one of which was *Wober das Leben?* (the origin of life), convinced *ignoramus et ignorabimus!*, meaning "we do not know and will not know!"

Since then, biological sciences came closer to solving a few of those riddles, but the origin of life remained a special case. Will we know, and how far have we come in the past 130 years? The origin of life cannot be discovered, it needs to be reinvented (25), meaning that the transition from the inorganic world to the organic world cannot be observed in nature anymore. Some day it may be reconstructed partly in the laboratory and possibly to some degree in vitro on the basis of an appropriate concept of ancestral metabolism. The phylogenetic reconstruction of ancestral metabolism in a general sense in turn requires the separation of life processes into parts that are absolutely essential, followed by trials to explain their abiotic formation, and finally by experimental attempts of in vitro reconstitution (63).

Regarding the essential parts, biologists allege that the biochemical unity that underlies the living world makes sense only if most of the central metabolic intermediates and pathways were already present in the common ancestor. This appears to be the blueprint of primordial metabolism: a network of a dozen common organic molecules (central precursor metabolites) from which all building blocks derive and which are transformed and interconnected by only a few reactions. I aim to document how variations of a few metabolic junctions between these common central metabolites resulted in autotrophic pathways.

The fixation of inorganic carbon into organic material (autotrophy) is a prerequisite for life and sets the starting point of biological evolution. In the extant biosphere the Calvin-Benson cycle is the predominant mechanism by which many prokaryotes and all plants fix $CO_2$ into biomass using the key enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). Yet, this cycle is not the only option. I briefly describe five alternative carbon fixation mechanisms that are found in prokaryotes. In view of the present dominance of the Calvin-Benson cycle, alternative pathways may be considered poor surrogates or even late stopgap solutions to replace this established route. This impression needs to be adjusted by detailed analysis of the decisive differences, the pros and cons of the alternative pathways. Confronted with the uncomfortable situation of finding six autotrophic pathways, everyone is seeking a rationale behind this diversity. I would like to stress that there are no good or bad solutions, as all pathways have stood the test of time for billions of years. In retrospect, taking the life conditions as a starting point and considering how the different autotrophic pathways adjust to them, one may receive an impression of evolution at work.

It is a widely accepted scenario that the decisive step toward the origin of life was the development of an autocatalytic mechanism by which inorganic volcanic gases were transformed, with the catalytic and structural help of minerals and metals, into those organic molecules that form the vital building blocks of all living things (109), notable of the constituents of a primordial RNA world (75). Most scenarios assume a strictly anaerobic and hot primordial world (69, 109, 110). Disputes on primordial metabolism led to opposite views, and often, models did not seriously take into consideration those metabolic strategies that are highly conserved in anaerobic prokaryotes. This review therefore advertises for the fascinating world of anaerobic autotrophic prokaryotes and their intriguing mechanisms of coupling energy-yielding chemical reactions of inorganic compounds to drive energy-consuming organic syntheses. Apart from connecting biosynthesis to energy metabolism, a structural framework (notably forming and energizing a membrane) and a primitive hereditary mechanism must be implemented concurrently to form a functional and viable unity. These aspects are beyond the scope of this review, as are industrial applications,

**Central precursor metabolites:** approximately a dozen metabolites of central pathways (such as acetyl-CoA, pyruvate, oxaloacetate, 2-oxoglutarate), from which all cellular building blocks are derived

**Calvin-Benson cycle:** most common cyclic carbon fixation pathway; named after its discoverers

**Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO):** the key carboxylase of the Calvin-Benson cycle that converts ribulose-1,5-bisphosphate plus $CO_2$ and water to two molecules of 3-phosphoglycerate

phylogenetic analyses of key enzymes, genetic organization, and regulation of pathways. Several recent reviews cover diverse aspects of autotrophic carbon fixation (12, 14, 24, 52, 78, 99).

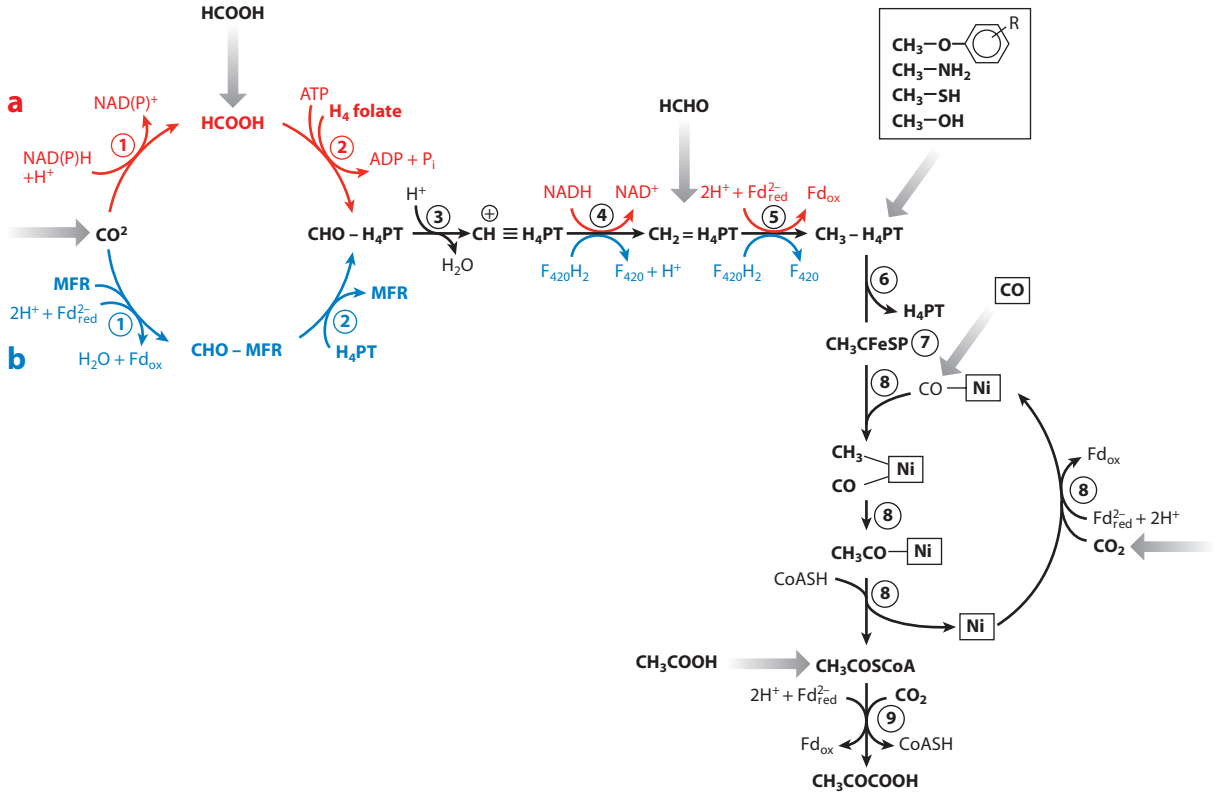
This review summarizes the five alternative pathways, their energetic aspects, their pros and cons in the real world, and what insights into the early evolution of life they might afford. The distribution of the pathways and their key features are briefly summarized in **Supplemental Table 1** (follow the **Supplemental Material link** from the Annual Reviews home page at **http://www.annualreviews.org**). **Supplemental Table 1** and **Figures 1–6** contain information about the reactions and enzymes of the pathways, the respective genes in model organisms that can be used for database search, as well as the free energy changes and equilibrium constants of the catalyzed reactions.

Supplemental Material



## REDUCTIVE ACETYL-COENZYME A PATHWAY

The reductive acetyl-coenzyme A (CoA) pathway (Wood-Ljungdahl pathway) was elucidated by the laboratories of Wood, Ljungdahl, Thauer, and others as a pathway that is used by acetogenic bacteria to synthesize acetyl-CoA from $CO_2$ (24, 67, 78, 114). It was later shown to operate in autotrophic $CO_2$ fixation in acetogenic and other Eubacteria and in methanogenic Euryarchaeota (23, 31, 32, 36, 52, 100, 108, 119). This is the only autotrophic pathway that can simultaneously fix $CO_2$ and generate ATP by converting acetyl-CoA to acetate. One molecule of $CO_2$ is reduced to the level of a methyl group, which is bound to a tetrahydropterin. Another $CO_2$ molecule is reduced to carbon monoxide and the combination of the CO and $CH_3$ groups with CoA yields acetyl-CoA (**Figure 1**).



## Enzymatic Steps in Acetogenic Bacteria

Most steps of $CO_2$ reduction to methyl-tetrahydrofolate ($H_4$folate) are involved in one-carbon metabolism in nearly all organisms, except $CO_2$ reduction to formate and formate activation by formyltetrahydrofolate synthetase. The following characteristics apply to *Moorella thermoacetica*, the best studied case (see Reference 78 and references cited therein). The first reaction is $CO_2$ (rather than $HCO_3^-$) reduction to formate with NADH catalyzed by formate dehydrogenase. 10-Formyl-tetrahydrofolate synthetase activates formate in an ATP-dependent condensation with $H_4$folate, forming 10-formyl-$H_4$folate. The next two steps are catalyzed by 5,10-methenyl-$H_4$folate cyclohydrolase and 5,10-methylene-$H_4$folate dehydrogenase, which may exist as separate entities or, more often, as a bifunctional enzyme cyclohydrolase-dehydrogenase. The bifunctional enzyme may protect the highly labile 5,10-methenyl-$H_4$folate from hydrolysis and channels it for reduction to the more stable 5,10-methylene-$H_4$folate. The last step is catalyzed by NADH- (or ferredoxin-)dependent 5,10-methylene-$H_4$folate reductase.

Methyl-$H_4$folate:corrinoid iron-sulfur protein methyltransferase catalyzes the transfer of the methyl group of methyl-$H_4$folate to the cobalt center of the heterodimeric corrinoid iron-sulfur protein. This reaction forms the first in a series of unique enzyme-bound

**Ferredoxin:** small proteins that contain one or several inorganic Fe-S clusters that function as electron carriers in redox reactions



**Figure 1**

Reductive acetyl-coenzyme A (CoA) pathway. The gray arrows show the entry of various one-carbon units. Here and in the following figure captions, the free energy changes under standard conditions, pH 7 ($\Delta G^{\circ\prime}$ reaction$^{-1}$), are given, as well as the associated equilibrium constants at pH 7, $K'_{eq}$, and the gene numbers of the enzymes (in parentheses). Note that the free energy change of 2 $CO_2$ + 4 $H_2$ + CoASH → $CH_3CO$-SCoA + 3 $H_2O$ ($\Delta G^{\circ\prime}$ –59 kJ) drops to +29 kJ if in vivo E′ for hydrogen drops to –300 mV, owing to a very low $H_2$ partial pressure. The redox potential $E^{\circ\prime}$ of ferredoxin in this and the other pathways was taken as –414 mV corresponding to that of the hydrogen/$H^+$ couple. Abbreviations: $Fd^{2-}_{red}$, reduced ferredoxin; $H_4$PT, tetrahydropterin; MFR, methanofuran; CFeSP, corrinoid-iron sulfur protein. (*a*) Pathway as studied in *Moorella thermoacetica* and related acetogenic bacteria (78). Reaction 1: Formate dehydrogenase (NADPH) ($\Delta G^{\circ\prime}$ +22 kJ; $K'_{eq}$ 1.4 × $10^{-4}$; Moth_2312/2314). Reaction 2: 10-Formyl-$H_4$ folate synthetase (ADP forming) ($\Delta G^{\circ\prime}$ –8 kJ; $K'_{eq}$ 25; Moth_0109). Reaction 3: 5,10-Methenyl-$H_4$ folate cyclohydrolase ($\Delta G^{\circ\prime}$ –4 kJ; $K'_{eq}$ 5; Moth_1516). Reaction 4: 5,10-Methylene-$H_4$ folate dehydrogenase [NAD(P)H] ($\Delta G^{\circ\prime}$ –3 kJ; $K'_{eq}$ 3; Moth_1516). Reaction 5: 5,10-Methylene-$H_4$ folate reductase ($Fd^{2-}_{red}$) ($\Delta G^{\circ\prime}$ –57 kJ; $K'_{eq}$ $10^{10}$ $M^2$; Moth_1191). Reaction 6: Methyl-$H_4$ folate: corrinoid iron-sulfur protein methyltransferase ($\Delta G^{\circ\prime}$ –4 kJ; $K'_{eq}$ 5; Moth_1197). Reaction 7: Corrinoid iron-sulfur protein (CFeSP) (Moth_1198/1201). Reaction 8: CO dehydrogenase/acetyl-CoA synthase ($Fd^{2-}_{red}$) [$CO_2$ + $Fd^{2-}_{red}$ + 2 $H^+$ + $CH_3$-CFeSP + CoASH → $CH_3CO$-SCoA + CFeSP + $Fd_{ox}$ + $H_2O$] ($\Delta G^{\circ\prime}$ 0 kJ; $K'_{eq}$ 1 $M^{-2}$; Moth_1198/1201/1202). Reaction 9: Pyruvate synthase ($Fd^{2-}_{red}$) ($\Delta G^{\circ\prime}$ +19 kJ; $K'_{eq}$ 5 × $10^{-4}$ $M^{-1}$; Moth_0064-). (*b*) The variant found in methanogenic Archaebacteria as studied in *Methanothermobacter marburgensis* (59, 104). Deazaflavin factor 420, $F_{420}$ ($E^{\circ\prime}$ –360 mV). Reaction 1: Formyl-methanofuran dehydrogenase ($Fd^{2-}_{red}$) ($\Delta G^{\circ\prime}$ +16 kJ; $K'_{eq}$ 1.5 × $10^{-3}$ $M^{-1}$; Mo enzyme MTBMA_c13060/13070(13050). Reaction 2: Formyl transferase ($\Delta G^{\circ\prime}$ –5 kJ; $K'_{eq}$ 7; MTBMA_c16460). Reaction 3: 5,10-Methenyl-tetrahydromethanopterin ($H_4$ methanopterin) cyclohydrolase ($\Delta G^{\circ\prime}$ –5 kJ; $K'_{eq}$ 7; MTBMA_c11690). Reaction 4: 5,10-Methylene-$H_4$-methanopterin dehydrogenase ($F_{420}H_2$) ($\Delta G^{\circ\prime}$ +6 kJ; $K'_{eq}$ 0.1; MTBMA_c00500). Reaction 5: 5,10-Methylene-$H_4$-methanopterin reductase ($F_{420}H_2$) ($\Delta G^{\circ\prime}$ –6 kJ; $K'_{eq}$ 10; MTBMA_c03270). Reaction 6: Methyl-$H_4$-methanopterin: corrinoid iron-sulfur protein methyltransferase ($\Delta G^{\circ\prime}$ –4 kJ; $K'_{eq}$ 5; MTBMA_c02920). Protein 7: Corrinoid iron-sulfur protein (MTBMA_c02910). Reaction 8: CO dehydrogenase (α MTBMA_c02870/14220/14210/14200; ε MTBMA_c14190/02880)/acetyl-CoA synthase (β MTBMA_c02890) ($Fd^{2-}_{red}$) ($\Delta G^{\circ\prime}$ 0 kJ; $K'_{eq}$ 1 $M^{-2}$). Reaction 9: Pyruvate synthase ($Fd^{2-}_{red}$) ($\Delta G^{\circ\prime}$ +19 kJ; $K'_{eq}$ 5 × $10^{-4}$ $M^{-1}$; MTBMA_c03130/03140/03150/03160). Standard free energy changes $\Delta G^{\circ\prime}$ were calculated in this work from the standard free energies of formation at 25°C (102), from reported equilibrium constants, or from standard redox potentials. Gases were taken in the gaseous state; all other compounds in the aqueous dissolved form. For the sake of simplicity, equations of ATP-consuming/ATP-forming reactions considered neither the participation of water nor the participation of charges and $H^+$ associated with these reactions, which refer to pH 7 ($K'_{eq}$). Note that $[H^+]$ in all equilibrium constants $K'_{eq}$ (calculated from $\Delta G^{\circ\prime}$ values referring to pH 7) and K′ needs to be set 1 (rather than $10^{-7}$ M) because pH 7 ($[H^+]$ = $10^{-7}$ M) was already taken into consideration in the $\Delta G^{\circ\prime}$ values. $[H_2O]$ in $K'_{eq}$ and K′ also needs to be set 1 (rather than 55 M), as the free energies of formation refer to compounds in the aqueous state (solutions in water). The free energy changes at cellular concentrations of the reactants $\Delta G'$ need to be corrected according to $\Delta G' = \Delta G^{\circ\prime} + RT \ln K'$, with $\Delta G^{\circ\prime} = -RT \ln K'_{eq}$.

bioorganometallic intermediates (methyl-Co, methyl-Ni, Ni-CO, acetyl-Ni). Cobalt in the corrinoid protein cycles between the Co(I) and methyl-Co(III) states during the reaction, with the Co(I) state required to initiate catalysis. This protein-bound Co(I) is highly reducing (mid potential −504 mV) and can easily undergo oxidative inactivation to the Co(II) state; this needs ATP- or adenosylmethionine-dependent resuscitation by transfer of one electron, e.g., from ferredoxin.

Formation of methyl-[Co(I)] is followed by methyl group transfer to a NiFeS cluster in acetyl-CoA synthase, which forms a complex with carbon monoxide dehydrogenases. Monofunctional Ni-CO dehydrogenase functions in oxidizing substrate CO. In contrast, bifunctional CO dehydrogenase/acetyl-CoA synthase (AcsAB) functions to reduce $CO_2$ to CO; the synthase also accepts the methyl group from the methylated corrinoid protein and converts CO, CoA, and the methyl group to acetyl-CoA, which is the precursor of cellular material and a source of energy (38, 78).

## Variations of the Scheme and Energetics

The bacterial variants are numerous. Formate dehydrogenase may function with ferredoxin and may contain either molybdenum or tungsten. Tetrahydrofolate, a pteroyl-monoglutamate, may be replaced by a polyglutamate form (65) or by methanopterins. Methylene-$H_4$folate reductase may use NAD(P)H instead of ferredoxin. The archaebacterial pathway (methanogens and *Archaeoglobus*) exhibits many special features and functional roles (**Figure 1**) (31, 78, 104). This machinery is also used for acetate conversion to $CH_4$ and $CO_2$ by various methanogens (28). One-carbon compounds enter the pathway as indicated in the figure. In this case, part of the $C_1$-$H_4$pterins needs to be oxidized to enable $CO_2$ reduction to CO. The pathway can even be reversed for the complete oxidation of acetyl-CoA, instead of the Krebs cycle (2, 88, 105).

Numerous acetogenic bacteria can grow autotrophically by reducing 2 $CO_2$ with 4 $H_2$ to acetate via acetyl-CoA. Acetyl-CoA conversion to acetate results in the generation of 1 ATP by substrate-level phosphorylation. However, 1 ATP is required for formate activation, leaving zero energy output unless the formation of acetyl-CoA from 2 $CO_2$ is coupled to electron transport phosphorylation; there is no site where substrate-level phosphorylation could occur. This problem is still unsolved and is complicated by the fact that acetogenic bacteria with (such as *Moorella thermoacetica*) and without (such as *Acetobacterium woodii*) cytochromes exist that may differ in energy conservation—very much like in methanogens (78, 104). In *A. woodii*, one of the steps between methylene-$H_4$folate and methyl-corrinoid protein (electron donors CO, $H_2$, or NADH) indeed generates a sodium motive force by an Rnf complex (42) (see below). In *M. thermoacetica* a kind of electron bifurcation associated with ferredoxin-dependent methylene-$H_4$folate reduction that is coupled to the reduction of a yet unknown heterodisulfide is conceivable; however, the mode of coupling to energy generation is unknown.

# REDUCTIVE CITRIC ACID CYCLE

The reductive citric acid cycle was discovered in the anaerobic *Chlorobium limicola*, a green phototrophic sulfur bacterium. It is named the Arnon-Buchanan cycle after its discoverers (27). This cycle presented the first exception to the presumed universal occurrence of the Calvin-Benson cycle and therefore has been refuted by the scientific community for years, despite convincing experimental evidence (35). The demonstration (56) and purification (3) of one of its missing key enzymes, ATP citrate lyase, have resolved the dispute (19). Meanwhile, this cycle was found in various groups of strict anaerobic and even microaerobic Eubacteria (4, 11, 12, 52, 89, 93).

This cycle is a reversal of the citric acid cycle (Krebs cycle), generating acetyl-CoA

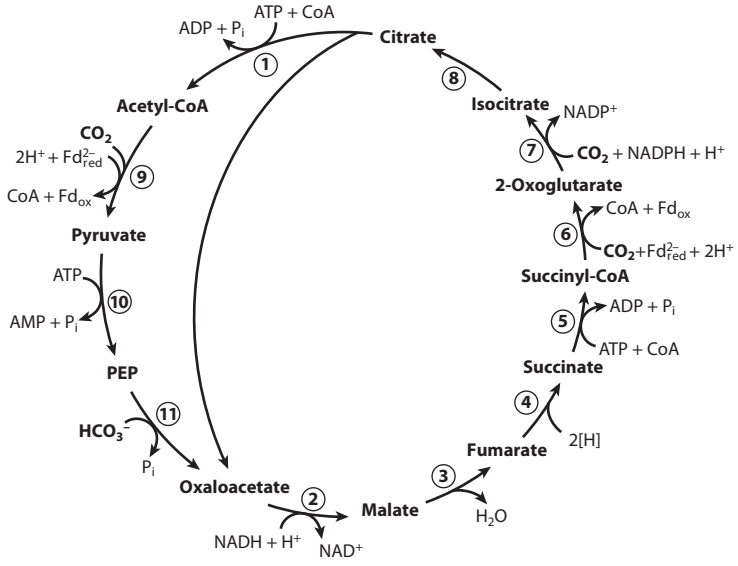



**Figure 2**

Reductive citric acid cycle. Enzymes as studied in *Hydrogenobacter thermophilus*. Reaction 1: Malate dehydrogenase (NADH) ($\Delta G^{\circ\prime}$ –30 kJ; $K'_{eq}$ 1.8 × $10^5$ $M^{-1}$; HTH_0756). Reaction 2: Fumarate hydratase ($\Delta G^{\circ\prime}$ +3 kJ; $K'_{eq}$ 0.3 M; HTH_0301). Reaction 3: Fumarate reductase ($\Delta G^{\circ\prime}$ –68 kJ assuming NADH as electron donor; $K'_{eq}$ $10^8$ $M^{-1}$; HTH_0983/1421). Reaction 4: Succinyl-CoA synthetase (ADP forming) ($\Delta G^{\circ\prime}$ +3 kJ; $K'_{eq}$ 0.3; HTH_1892/1893). Reaction 5: 2-Oxoglutarate synthase ($Fd^{2-}_{red}$) ($\Delta G^{\circ\prime}$ +19 kJ; $K'_{eq}$ 5 × $10^{-4}$ $M^{-1}$; HTH_1094/1095/1096/1097/1098/1099). Reaction 6a: 2-Oxoglutarate carboxylase (ADP forming) (HTH_1392/1393) + Reaction 6b: Isocitrate dehydrogenase (nondecarboxylating) (NADPH) (HTH_0836) ($\Delta G^{\circ\prime}$ –25 kJ; $K'_{eq}$ 2 × $10^4$), or Reaction 6: isocitrate dehydrogenase [NAD(P)H] (decarboxylating) in many other bacteria ($\Delta G^{\circ\prime}$ +8 kJ; $K'_{eq}$ 0.04 $M^{-1}$). Reaction 7: Aconitase ($\Delta G^{\circ\prime}$ –7 kJ; $K'_{eq}$ 17; HTH_0755). Reaction 8a: Citryl-CoA synthetase (HTH_0201/1737) + Reaction 8b: Citryl-CoA lyase (HTH_0311), or ATP citrate (*pro3S*)-lyase in many other bacteria ($\Delta G^{\circ\prime}$ +4 kJ; $K'_{eq}$ 0.2). Reaction 9: Pyruvate synthase ($Fd^{2-}_{red}$) ($\Delta G^{\circ\prime}$ +19 kJ; $K'_{eq}$ 5 × $10^{-4}$ $M^{-1}$; HTH_1551/1552/1553/1554/1555). Reaction 10: Pyruvate:water dikinase [phosphoenolpyruvate (PEP) synthetase] (AMP + $P_i$ forming) ($\Delta G^{\circ\prime}$ –12 kJ; $K'_{eq}$ 120; HTH_1644). Reaction 11: PEP carboxylase ($\Delta G^{\circ\prime}$ –24 kJ; $K'_{eq}$ 1.6 × $10^4$). In *H. thermophilus*, pyruvate carboxylase is active ($pyruvate^- + HCO_3^- + ATP \rightarrow oxaloacetate^{2-} + H_2O + ADP + P_i$; $\Delta G^{\circ\prime}$ –5 kJ; $K'_{eq}$ 8 M; HTH_1452/1598). Overall equation: 2 $CO_2$ + 3 NAD(P)H + 5 $H^+$ + $Fd^{2-}_{red}$ + CoASH + 2 ATP → $CH_3CO\text{-}SCoA$ + 3 $NAD(P)^+$ + $Fd_{ox}$ + 3 $H_2O$ + 2 ADP + 2 $P_i$; $\Delta G^{\circ\prime}$ –68 kJ [assuming normal NAD(P)H-dependent isocitrate dehydrogenase is active and fumarate reductase also works with NAD(P)H]. Abbreviation: $Fd^{2-}_{red}$, reduced ferredoxin.

from 2 $CO_2$ rather than inversely oxidizing acetyl-CoA (**Figure 2**). However, several irreversible steps of the Krebs cycle need to be modified to turn the cycle into the reductive direction. Succinate dehydrogenase is replaced by fumarate reductase. $NAD^+$-dependent 2-oxoglutarate dehydrogenase is replaced by ferredoxin-dependent 2-oxoglutarate synthase (2-oxoglutarate:ferredoxin oxidoreductase). Citrate synthase is replaced by ATP citrate lyase. The overall reactions from succinate up to acetyl-CoA and oxaloacetate are endergonic ($\Delta G^{\circ\prime}$ +27 kJ), which causes problems, notably because succinyl-CoA is labile. Thermophilic *Hydrogenobacter* and *Aquifex* species therefore replaced isocitrate dehydrogenase by a two-component system that favors isocitrate formation. It consists of an ATP- and biotin-dependent 2-oxoglutarate carboxylase resembling pyruvate carboxylase

**Thermophilic:** describes organisms growing at temperatures exceeding by far the ambient temperature

that forms oxalosuccinate and a nondecarboxylating isocitrate dehydrogenase that reduces oxalosuccinate to isocitrate (5, 8).

Reduced ferredoxin is required for 2-oxoglutarate synthase; this reaction is unfavorable and requires input of energy to keep ferredoxin reduced (see below). This problem of keeping ferredoxin reduced applies also to the reductive carboxylation of acetyl-CoA by pyruvate synthase. There may be two forms of 2-oxoacid synthases with different oxygen sensitivity (115, 116). Fumarate reductase also exists in several forms: thiol dependent (in methanogens with an incomplete reductive citric acid cycle) (41), NADH dependent (72), or electron donor unknown and in vitro working with reduced viologen dyes (80, 82). ATP citrate lyase may be either a one-enzyme or a two-enzyme system comprising a subunit that synthesizes and cleaves citryl-CoA (3, 6, 7). Some bacteria that might harbor the reductive citric acid cycle do not contain an obvious gene for ATP citrate lyase, raising suspicion that another type of this enzyme might exist (52). Some facultative, strict anaerobes reverse the cycle for the complete oxidation of acetyl-CoA, when a suitable organic substrate and an electron acceptor such as sulfate are present (89, 105). This allows for the synthesis of another ATP by ATP citrate lyase as well as for the generation of reduced ferredoxin by 2-oxoglutarate synthase; oxidation of reduced ferredoxin by $NAD^+$ in turn may generate a proton motive force (see below).

## DICARBOXYLATE/ 4-HYDROXYBUTYRATE CYCLE

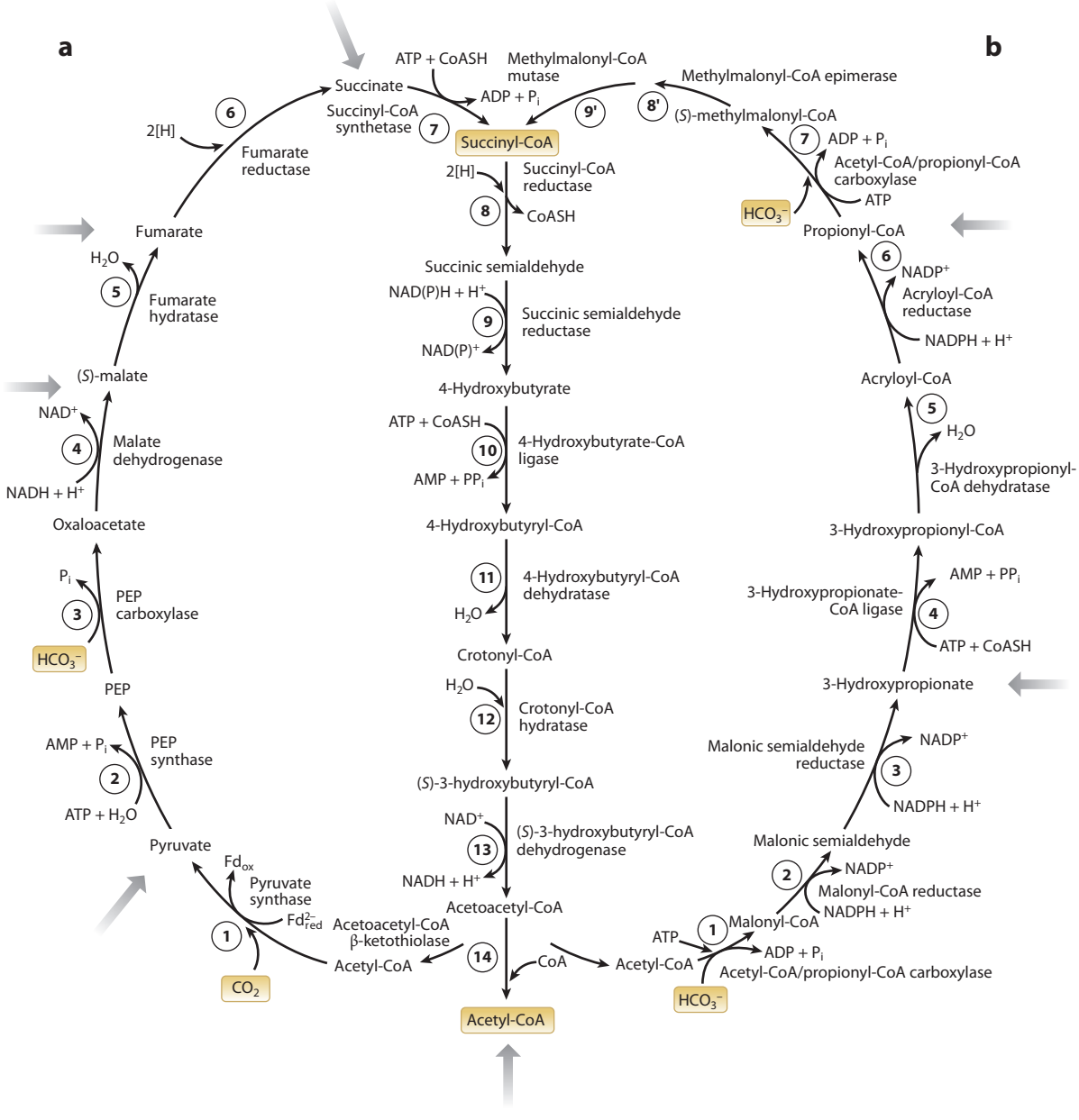
This cycle functions in the anaerobic autotrophic members of the *Thermoproteales* and *Desulfurococcales* (Crenarchaeota) (48, 80, 82) (**Figure 3*a***), but also in *Pyrolobus fumarii*

**Figure 3**

Dicarboxylate/4-hydroxybutyrate cycle and 3-hydroxypropionate/4-hydroxybutyrate cycle. The gray arrows show the entry of various organic substrates. Note that the $\Delta G^{\circ\prime}$ and $K'_{eq}$ values do not refer to the high growth temperatures of the organisms.
(*a*) Dicarboxylate/4-hydroxybutyrate cycle. Enzymes as studied in *Thermoproteus neutrophilus*. Reaction 1: Pyruvate synthase ($Fd^{2-}_{red}$) ($\Delta G^{\circ\prime}$ +19 kJ; $K'_{eq}$ 0.0005 $M^{-1}$; candidate genes Tneu_0130/0131; Tneu_0176/0177/0178/0179; Tneu_1526/1527/1528/1529; Tneu_1795/1796/1797). Reaction 2: phosphoenolpyruvate (PEP) synthetase (AMP + $P_i$ forming) ($\Delta G^{\circ\prime}$ –12 kJ; $K'_{eq}$ 120; Tneu_1204). Reaction 3: PEP carboxylase ($\Delta G^{\circ\prime}$ –24 kJ; $K'_{eq}$ 1.6 × $10^4$; Tneu_0418). Reaction 4: Malate dehydrogenase (NADH) ($\Delta G^{\circ\prime}$ –30 kJ; $K'_{eq}$ 1.8 × $10^5$ $M^{-1}$; Tneu_1509). Reaction 5: Fumarate hydratase ($\Delta G^{\circ\prime}$ +3 kJ; $K'_{eq}$ 0.3 M; Tneu_1334/1335). Reaction 6: Fumarate reductase, electron donor unknown ($\Delta G^{\circ\prime}$ –68 kJ; $K'_{eq}$ 8 × $10^{11}$; Tneu_0423/0424). Reaction 7: Succinyl-CoA synthetase (ADP forming) ($\Delta G^{\circ\prime}$ +3 kJ; $K'_{eq}$ 0.3; Tneu_1463/1464). Reaction 8: Succinyl-CoA reductase (NADPH) ($\Delta G^{\circ\prime}$ +12 kJ; $K'_{eq}$ 0.008; Msed_0709/Tneu_0421). Reaction 9: Succinic semialdehyde reductase (NADPH) ($\Delta G^{\circ\prime}$ –24 kJ; $K'_{eq}$ 1.5 × $10^4$ $M^{-1}$; Msed_1424/Tneu_0419). Reaction 10: 4-Hydroxybutyrate-CoA ligase (AMP forming) ($\Delta G^{\circ\prime}$ –6 kJ; $K'_{eq}$ 11; gene unknown in *Metallosphaera sedula*/Tneu_0420). Reaction 11: 4-Hydroxybutyryl-CoA dehydratase ($\Delta G^{\circ\prime}$ –3 kJ; $K'_{eq}$ 3 M; Msed_1321/Tneu_0422). Reaction 12: Crotonyl-CoA hydratase ($\Delta G^{\circ\prime}$ –3 kJ; $K'_{eq}$ 3 $M^{-1}$; Msed_0399/Tneu_0541). Reaction 13: (*S*)-3-hydroxybutyryl-CoA dehydrogenase ($NAD^+$) ($\Delta G^{\circ\prime}$ +15 kJ; $K'_{eq}$ 0.002 M; Msed_0399/Tneu_0541). Reaction 14: Acetoacetyl-CoA β-ketothiolase ($\Delta G^{\circ\prime}$ –27 kJ; $K'_{eq}$ 5 × $10^4$ $M^{-1}$; Msed_0656/Tneu_0249). (*b*) 3-Hydroxypropionate/ 4-hydroxybutyrate cycle. Enzymes, as studied in *M. sedula*. Reaction 1: Acetyl-CoA carboxylase (ADP forming) ($\Delta G^{\circ\prime}$ –14 kJ; $K'_{eq}$ 280 M; Msed_0147/0148/1375). Reaction 2: Malonyl-CoA reductase (NADPH) ($\Delta G^{\circ\prime}$ +10 kJ; $K'_{eq}$ 0.02; Msed_0709). Reaction 3: Malonic semi-aldehyde reductase (NADPH) ($\Delta G^{\circ\prime}$ –24 kJ; $K'_{eq}$ 1.6 × $10^4$ $M^{-1}$; Msed_1993). Reaction 4: 3-Hydroxypropionyl-CoA synthetase (AMP forming) ($\Delta G^{\circ\prime}$ –6 kJ; $K'_{eq}$ 11; Msed_1456). Reaction 5: 3-Hydroxypropionyl-CoA dehydratase ($\Delta G^{\circ\prime}$ –5 kJ; $K'_{eq}$ 7 M; Msed_2001). Reaction 6: Acryloyl-CoA reductase (NADPH) ($\Delta G^{\circ\prime}$ –50 kJ; $K'_{eq}$ 6 × $10^8$ $M^{-1}$; Msed_1426). Reaction 7: Propionyl-CoA carboxylase (ADP forming) ($\Delta G^{\circ\prime}$ –11 kJ; $K'_{eq}$ 120 M; Msed_0147/0148/1375). Reaction 8′: Methylmalonyl-CoA epimerase ($\Delta G^{\circ\prime}$ 0 kJ; $K'_{eq}$ 1; Msed_0639). Reaction 9′: Methylmalonyl-CoA mutase ($\Delta G^{\circ\prime}$ –7 kJ; $K'_{eq}$ 20; Msed_0638/2055); succinyl-CoA conversion to 2 acetyl-CoA as in *T. neutrophilus*. Note that the equilibrium of a reaction may be far on the product side in vivo even if the free energy change under standard conditions is positive. For instance, Reaction 2 in panel *b* ($\Delta G^{\circ\prime}$ +10 kJ) has a $K'_{eq}$ [malonic semialdehyde] [$NADP^+$] [CoASH]/[malonyl-SCoA] [NADPH] [$H^+$] value of approximately 0.02. However, assuming cellular concentrations, for instance, 0.12 mM NADPH, 0.002 mM $NADP^+$, and 1.4 mM CoASH ([$H^+$] set 1 because $K_{eq}$ derived from $\Delta G^{\circ\prime}$ was already corrected for pH 7), the resulting ratio of [malonic semialdehyde]/[malonyl-SCoA] at equilibrium under in vivo conditions ($\Delta G' = 0$) would be approximately 700/1.
Abbreviation: $Fd^{2-}_{red}$, reduced ferredoxin.

(*Desulfurococcales*) (15), which has adapted to a facultative aerobic energy metabolism at low oxygen pressure.

The cycle can be divided into two parts. In the first part, acetyl-CoA, one $CO_2$, and one bicarbonate are transformed via $C_4$ dicarboxylic acids to succinyl-CoA. In the second part, succinyl-CoA is converted via 4-hydroxybutyrate to two molecules of acetyl-CoA. One acetyl-CoA can be used for biosynthesis and the other serves as a $CO_2$ acceptor for the next round of the cycle. The cycle starts with the reductive carboxylation of acetyl-CoA to pyruvate catalyzed by pyruvate synthase. Pyruvate is converted to phosphoenolpyruvate (PEP), followed by carboxylation of PEP to



oxaloacetate catalyzed by an archaebacterial PEP carboxylase (76). The subsequent oxaloacetate reduction involves an incomplete reductive citric acid cycle that stops at succinyl-CoA; it is not complete as originally thought (97).

Succinyl-CoA is further reduced to succinic semialdehyde and then to 4-hydroxybutyrate. The latter compound is converted to two acetyl-CoA molecules, which requires the key enzyme 4-hydroxybutyryl-CoA dehydratase. It is a [4Fe-4S] cluster- and FAD-containing enzyme that catalyzes the elimination of water from 4-hydroxybutyryl-CoA by a ketyl radical mechanism (70). Its product, crotonyl-CoA, is converted to two molecules of acetyl-CoA via normal β-oxidation reactions. The regulation of the cycle was studied in *T. neutrophilus* (81).

## 3-HYDROXYPROPIONATE/ 4-HYDROXYBUTYRATE CYCLE

This cycle functions in aerobic autotrophic *Sulfolobales* (Crenarchaeota, Archaebacteria) (9, 13, 49, 61, 82), including the strictly anaerobic *Stygiolobus azoricus*, which has returned to an anaerobic lifestyle (**Figure 3*b***) (15). The enzymes of the cycle are oxygen tolerant, as required for life with oxygen. One of its key enzymes, 4-hydroxybutyryl-CoA dehydratase, is involved in γ-aminobutyrate fermentation in clostridia (90), where it is inactivated by oxygen. In *Sulfolobales*, 4-hydroxybutyryl-CoA dehydratase is sufficiently oxygen insensitive to operate under microoxic or even oxic conditions (15). The presence of genes coding for key enzymes of the 3-hydroxypropionate/ 4-hydroxybutyrate cycle in the mesophilic marine group I Crenarchaeota, such as *Cenarchaeum symbiosum* and *Nitrosopumilus maritimus* ("Thaumarchaeota"; 18) (13, 39, 111), suggests that these and related abundant marine Archaebacteria (58) as well as related soil species (120) may use a similar cycle.

In the cycle, one molecule of acetyl-CoA is formed from two molecules of bicarbonate. The carboxylating enzyme is the bifunctional biotin-dependent acetyl-CoA/propionyl-CoA carboxylase (20, 21, 50, 74). The cycle can also be divided into two parts. The first part transforms acetyl-CoA and two bicarbonate molecules via 3-hydroxypropionate to succinyl-CoA (13, 53, 71), as described below for the 3-hydroxypropionate bi-cycle. However, the enzymes used to synthesize propionyl-CoA from malonyl-CoA are not homologous, although the intermediates are the same (13, 117). Therefore, these pathways that, examined superficially, are seen as similar have evolved independently in *Sulfolobales* and *Chloroflexi*. The second part, which apparently is common to the autotrophic Crenarchaeota, converts succinyl-CoA via 4-hydroxybutyrate to two acetyl-CoA molecules. Pyruvate is formed from succinyl-CoA via oxidative decarboxylation of malate by malic enzyme, which requires one and a half turns of the cycle to build succinyl-CoA from four molecules of bicarbonate (26).

## 3-HYDROXYPROPIONATE BI-CYCLE

The phototrophic green nonsulfur bacterium *Chloroflexus aurantiacus* and related *Chloroflexi* use the 3-hydroxypropionate bi-cycle for autotrophic $CO_2$ fixation (45, 46, 98, 117). Note, however, that *Oscillochloris trichoides* harbors the Calvin-Benson cycle (55). The pathway starts from acetyl-CoA, and conventional ATP- and biotin-dependent acetyl-CoA and propionyl-CoA carboxylases act as carboxylating enzymes.

Each turn of the first cycle results in the net fixation of two molecules of bicarbonate into one molecule of glyoxylate (**Figure 4**). Acetyl-CoA is carboxylated to malonyl-CoA by acetyl-CoA carboxylase. The reduction of malonyl-CoA to propionyl-CoA is catalyzed by only two enzymes, the bifunctional malonyl-CoA reductase (51) and the trifunctional propionyl-CoA synthase (1). Propionyl-CoA is carboxylated to methylmalonyl-CoA followed by isomerization to succinyl-CoA; these reactions are used in many organisms for propionate assimilation. Note that these steps are identical to those of the first part of the 3-hydroxypropionate/4-hydroxybutyrate

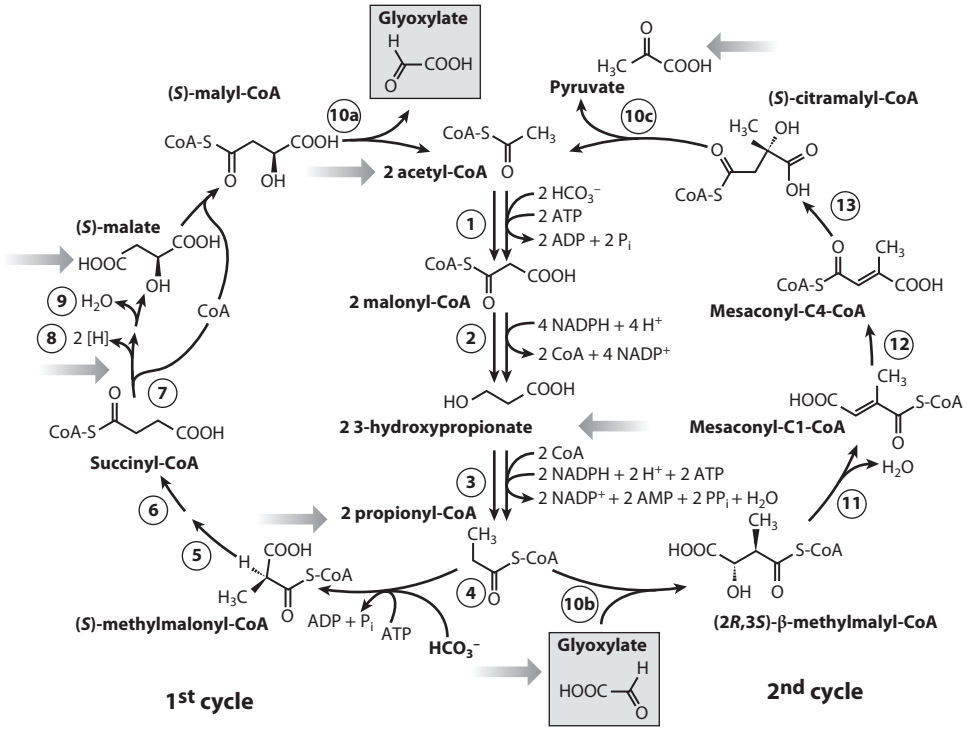



**Figure 4**

3-Hydroxypropionate bi-cycle. The gray arrows show the entry of various organic substrates. Enzymes as studied in *Chloroflexus aurantiacus*. Reaction 1: Acetyl-CoA carboxylase (ADP forming) ($\Delta G^{\circ\prime}$ –14 kJ; $K'_{eq}$ 280 M; candidate genes Caur_1378/1647/1648/2034/2360/2832/3421/3739). Reaction 2: Malonyl-CoA reductase (NADPH) ($\Delta G^{\circ\prime}$ –14 kJ; $K'_{eq}$ 280 $M^{-1}$; Caur_2614). Reaction 3: Propionyl-CoA synthase (AMP forming, NADPH) ($\Delta G^{\circ\prime}$ –61 kJ; $K'_{eq}$ 5 × $10^{10}$; Caur_0613). Reaction 4: Propionyl-CoA carboxylase (ADP forming) ($\Delta G^{\circ\prime}$ –11 kJ; $K'_{eq}$ 120; candidate genes Caur_1378/1647/1648/2034/2360/2832/3421/3739). Reaction 5: Methylmalonyl-CoA epimerase ($\Delta G^{\circ\prime}$ 0 kJ; $K'_{eq}$ 1; Caur_3037). Reaction 6: Methylmalonyl-CoA mutase ($\Delta G^{\circ\prime}$ –7 kJ; $K'_{eq}$ 20; Caur_2508/2509). Reaction 7: Succinyl-CoA:(*S*)-malate-CoA transferase ($\Delta G^{\circ\prime}$ 0 kJ; $K'_{eq}$ 1; Caur_0178/0179). Reaction 8: Succinate dehydrogenase (quinone) ($\Delta G^{\circ\prime}$ +20 kJ; $K'_{eq}$ 0.0004; Caur_1880/1881/1882). Reaction 9:, Fumarate hydratase ($\Delta G^{\circ\prime}$ –3 kJ; $K'_{eq}$ 3 $M^{-1}$; Caur_1443). Reaction 10a: (*S*)-malyl-CoA lyase ($\Delta G^{\circ\prime}$ –19 kJ; $K'_{eq}$ 2.1 × $10^3$ M; Caur_0174). Reaction 10b: β-Methylmalyl-CoA lyase ($\Delta G^{\circ\prime}$ +18 kJ; $K'_{eq}$ 0.0007 $M^{-1}$; Caur_0174). Reaction 11: Mesaconyl-C1-CoA hydratase (β-methylmalyl-CoA dehydratase) ($\Delta G^{\circ\prime}$ –6 kJ; $K'_{eq}$ 10 M; Caur_0173). Reaction 12: Mesaconyl-CoA C1-C4 CoA transferase ($\Delta G^{\circ\prime}$ 0 kJ; $K'_{eq}$ 1; Caur_0175). Reaction 13: Mesaconyl-C4-CoA hydratase ($\Delta G^{\circ\prime}$ –6 kJ; $K'_{eq}$ 14 $M^{-1}$; Caur_0180); Reaction 10c: (*S*)-citramalyl-CoA lyase ($\Delta G^{\circ\prime}$ –20 kJ; $K'_{eq}$ 3 × $10^3$ M; Caur_0174).

cycle. Succinyl-CoA is used for (*S*)-malate activation by CoA transfer, forming succinate and (*S*)-malyl-CoA. Succinate in turn is oxidized to (*S*)-malate, and in the last step (*S*)-malyl-CoA is cleaved into acetyl-CoA and glyoxylate (98). Acetyl-CoA can serve as starter for another round, and glyoxylate is assimilated in a second cycle (therefore the term bi-cycle).

In the second cycle, glyoxylate is combined with propionyl-CoA (an intermediate of the first cycle) to form β-methylmalyl-CoA (44, 45, 117). This condensation is followed by a series of $C_5$-transforming reactions that yield (*S*)-citramalyl-CoA. (*S*)-citramalyl-CoA is cleaved into acetyl-CoA and pyruvate by a trifunctional lyase, which before cleaved

(*S*)-malyl-CoA and formed β-methylmalyl-CoA. Thus, the puzzling disproportionation of glyoxylate and propionyl-CoA into acetyl-CoA and pyruvate without any redox reaction is solved in an elegant and economic way requiring only three additional enzymes (117). The whole bi-cyclic pathway results in pyruvate formation from three molecules of bicarbonate and involves 19 steps, but only 13 enzymes.

## RuBisCO IN ARCHAEBACTERIA

It is quite possible that even more autotrophic pathways exist. One conflicting result concerns the Calvin-Benson cycle, which appears not to operate in Archaebacteria. Yet, various Archaebacteria contain a gene for a distinct archaebacterial RuBisCO type III, which is expressed as a functional enzyme; it is found in autotrophic and heterotrophic as well as anaerobic and aerobic species (29, 62, 68, 79, 86). The apparently haphazard distribution of the gene makes explanations of its function more difficult. The classical route of ribulose-1,5-bisphosphate regeneration is lacking; two hypotheses have been put forward. Ribulose-1,5-bisphosphate may be formed via ribose-1,5-bisphosphate either from 5-phosphoribose-1-pyrophosphate (30) or from AMP (86). A role in $O_2$ detoxification or in consumption of excess reducing equivalents is less likely because the enzyme is also found in aerobes. The function of archaebacterial RuBisCO remains an intriguing issue.

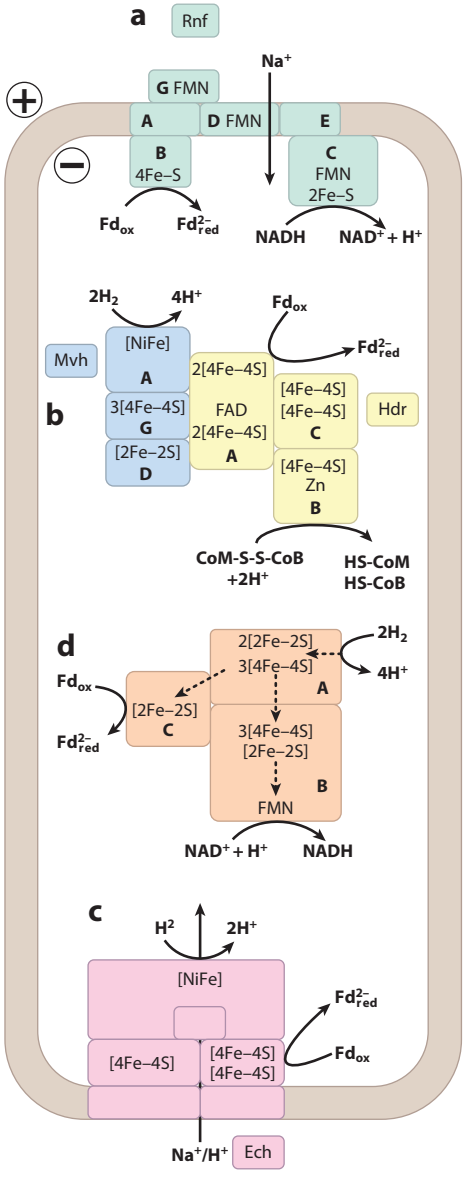
## HOW CAN CHEMOLITHO-AUTOTROPHIC ANAEROBES KEEP FERREDOXIN REDUCED?

Three of the six autotrophic pathways are operating under anaerobic (or microaerobic) conditions, and all of them involve highly oxygen-sensitive reduction steps that are driven by reduced ferredoxin. These reduction steps include formate dehydrogenase (Eubacteria) ($E^{\circ\prime}$ –420 mV) or formylmethanofuran dehydrogenase (methanogenic Archaebacteria) ($E^{\circ\prime}$ –500 mV), CO dehydrogenase/acetyl-CoA synthase (also called decarbonylase/acetyl-CoA synthase) ($E^{\circ\prime}$ –520 mV), pyruvate synthase ($E^{\circ\prime}$ –500 mV), and 2-oxoglutarate synthase ($E^{\circ\prime}$ –500 mV). In addition, even many heterotrophic anaerobes need pyruvate synthase and thus reduced ferredoxin for the assimilation of not only acetate, but also of all other substrates that are metabolized via acetyl-CoA. The standard redox potentials of these (reductive carboxylation) reactions are on the order of –500 mV, which is obviously more negative than that of $H_2$ (–414 mV) and of most ferredoxins (between –400 and –450 mV, abbreviated in the following equations as Fd). Actually, exceptional ferredoxins may have a midpoint potential more negative than –450 mV; still, they need to be reduced by inorganic electron donors with a more positive redox potential. How can this be accomplished? Some answers to this intriguing and long-lasting question were obtained only recently (**Figure 5**). Future experimental studies, guided by genome analysis of autotrophic bacteria, are needed to determine which of these mechanisms are used in the different anaerobic or microaerobic autotrophs and whether even new principles are realized.

### A Telling Example

A surprising experimental outcome illustrates the energetic problem. When *Methanothermobacter marburgensis* growing autotrophically with $H_2$ and $CO_2$ was fed with trace amounts of $^{14}$C-labeled acetate, cells incorporated $^{14}$C equally into acetyl units and pyruvate. This was to be expected if pyruvate synthase catalyzes the reductive carboxylation of acetyl-CoA to pyruvate. Feeding [2–$^{14}$C]pyruvate resulted in labeled pyruvate, but acetyl units were almost unlabeled (34). This counterintuitive result indicated that in vivo the equilibrium of (reversible) pyruvate synthesis from acetyl-CoA and $CO_2$ ($E^{\circ\prime}$ –500 mV, $\Delta G^{\circ\prime}$ + 19.2 kJ/mol with $H_2$) was far on the side of pyruvate. Such an endergonic reaction cannot be driven simply by ferredoxin with a standard redox potential near that of hydrogen. This means that energy must be spent to keep ferredoxin in vivo almost completely in the reduced form to reach a

potential of –500 mV or even lower. Even if pyruvate synthase reaction were at equilibrium ($\Delta G' = 0$) in vivo and all other reactants (acetyl-CoA, $CO_2$, CoASH, pyruvate) were present at a concentration of 1 mM, the resulting ratio of $[Fd^{2-}_{red}]/[Fd_{ox}]$ would be 2,000/1. It requires some sort of energy input to create this disequilibrium. The amount of energy needed is less than that of ATP hydrolysis ($\Delta G^{\circ\prime}$ –31.8 kJ), but ATP is not involved.




How can chemolithoautotrophic anaerobes keep ferredoxin with mid-potentials between –400 and –500 mV fairly reduced by use of hydrogen as electron donor? Originally, it was thought that in methanogens the reduced electron carrier deazaflavin Factor 420 ($E^{\circ\prime}$ –360 mV) could provide low-potential electrons by disproportionation of the deazaflavin radical: Reduced deazaflavin would donate low-potential electrons generating the semiquinone radical (2 deazaflavin$H_2$ → 2 deazaflavinH + 2 $H^+$ + 2 $e^-$, $E^{\circ\prime}$ –770 mV) to drive the reductive carboxylation of acetyl-CoA forward ($E^{\circ\prime}$ –500 mV), whereas the high-potential electrons (2 deazaflavinH → 2 deazaflavin + 2 $H^+$ + 2 $e^-$, $E^{\circ\prime}$ –50 mV) are consumed in the course of fumarate reduction to succinate ($E^{\circ\prime}$ +33 mV) [(34); note that this organism requires fumarate reductase to form 2-oxoglutarate via an incomplete reductive citric acid cycle (33), which, however, turned out to function with thiols rather than with deazaflavin (41)]. As seen below, a type of electron bifurcation in fact exists, but on the basis of FAD instead of deazaflavin, with the participation of ferredoxin, and using a positive electron acceptor other than fumarate.

**Chemolithoautotrophy:** a system in which energy is derived from a chemical reaction (chemotrophic) based on inorganic substrates (lithotrophic), and the carbon source, $CO_2$, is inorganic as well (autotrophic = self-nourishing)

## Situation in Chemolithoautotrophs and Photolithoautotrophs

The complexity of the problem of energy coupling can be best visualized by considering the

**Figure 5**

Mechanisms that can bring about the reduction of ferredoxin with NADH or with $H_2$ at ambient partial pressure. (*a*) Ferredoxin reduction by NADH by Rnf (*Rhodobacter* nitrogen fixation) complex composed of at least six different subunits. The predicted functions remain to be experimentally validated. (*b*) Ferredoxin reduction by $H_2$ in methanogens by soluble [NiFe]-hydrogenase associated with heterodisulfide reductase (via electron bifurcation). (*c*) Ferredoxin reduction by $H_2$ in methanogens and other anaerobes by membrane-bound energy-converting [NiFe]-hydrogenases (via chemiosmotic coupling). (*d*) Ferredoxin reduction by $H_2$ by soluble bifurcating (Fe-Fe) hydrogenases (hypothetical).

energetic situation of autotrophic methanogens that reduce $CO_2$ with 4 $H_2$ to $CH_4$ + 2 $H_2O$ to generate ATP (104). Part of the $CH_3$ intermediate, methyl-tetrahydromethanopterin, is withdrawn for the synthesis of acetyl-CoA, the precursor of cell carbon, which links autotrophic carbon fixation to energy metabolism. Under standard conditions, the reduction of ferredoxin with $H_2$ is near equilibrium, whereas under in vivo conditions ($pH_2$ = 10 Pa; pH 7; $E^{\circ\prime}$ of the $H_2/H^+$ couple –300 mV) it is strongly endergonic. It should be added that virtually fully reduced ferredoxin is required for the reduction of $CO_2$ to formylmethanofuran (the first $CO_2$ fixation step; $E^{\circ\prime}$ –500 mV) and for additional unfavorable reactions. All these ferredoxin-dependent reduction steps are catalyzed by cytoplasmic enzymes. Therefore, it is the reduction of ferredoxin with $H_2$ that must be energy driven, and the cytoplasmic membrane is probably the site of energy-driven reduction of ferredoxin (103, 104).

In photoautotrophic bacteria, the situation is much less complex. In anoxygenic photosynthesis, photosystem I reduces ferredoxin. In photosystem II, electrons arrive at the quinone level and need to be lifted to the level of NAD(P)H via light-driven reverse electron transport. Anoxygenic phototrophs that use the Calvin-Benson cycle or the 3-hydroxypropionate bi-cycle do not need to generate reduced ferredoxin except when fixing nitrogen.

## ENERGY-DRIVEN REDUCTION OF FERREDOXIN

First we discuss two mechanisms by which ferredoxin can be reduced by NADH.

### Ferredoxin Reduction by NADH: Membrane-Bound Rnf Complex

Rnf (*Rhodobacter* nitrogen fixation) was first discovered in the purple nonsulfur bacterium *Rhodobacter capsulatus*, where it is involved in supplying reduced ferredoxin for nitrogen fixation (57, 91). In *Acetobacterium woodii*, the *rnf* gene cluster encodes an energy-conserving ferredoxin:$NAD^+$ oxidoreductase complex (16, 17). In heterotrophically grown cells, it catalyzes the oxidation of reduced ferredoxin coupled with the reduction of $NAD^+$, and this process is coupled with the electrogenic transduction of $Na^+$ ions across the membrane to the outside.

$$Fd^{2-}_{red} + NAD^+ + H^+ \leftrightarrow NADH + Fd_{ox} + \Delta\mu Na^+.$$

Related complexes in Eubacteria generate a sodium motive force in the course of NADH oxidation coupled to ubiquinone reduction ($Na^+$-translocating NADH:quinone oxidoreductase Nqr) (107). In most autotrophs, the enzyme may operate in the reverse direction using an energized membrane to drive ferredoxin reduction with NADH at the cost of a sodium (or sometimes proton) motive force (**Figure 5*a***).

$$NADH + Fd_{ox} + \Delta\mu Na^+ \leftrightarrow Fd^{2-}_{red} + NAD^+ + H^+.$$

Hence, the Rnf system, which is present in many Eubacteria and a few Archaebacteria, represents a unique type of primary $Na^+$ pump and an early evolutionary mechanism of energy conservation that expands the redox range known to support life. It gives a mechanistic explanation for the lifestyle of many anaerobic prokaryotes and for the energetic driving force for the endergonic reduction of ferredoxin with NADH (16, 17).

### Ferredoxin Reduction by NADH: Soluble Electron-Bifurcating Enzymes

Another elegant strategy of reducing ferredoxin with NAD(P)H, called electron bifurcation, was recently discovered in *Clostridium kluyveri* (43, 66). The cytoplasmic butyryl-CoA dehydrogenase complex couples the endergonic reduction of ferredoxin ($E^{\circ\prime}$ –420 mV) with NADH ($E^{\circ\prime}$ –320 mV) to the exergonic reduction of crotonyl-CoA to butyryl-CoA ($E^{\circ\prime}$ –10 mV) with another NADH. This enzyme is the prototype of a mechanism used by

autotrophic anaerobes to generate reduced ferredoxin (or extra NADPH; 112). The electron flow from 2 NADH ($NADH/NAD^+$, $E^{\circ\prime}$ –320 mV) through 2 $FADH_2$ ($FADH_2/FAD$, $E^{\circ\prime}$ –240 mV) is bifurcated such that the oxidation of 2 $FADH_2$ to 2 FADH ($FADH_2/FADH$, $E^{\circ\prime}$ –400 mV) is coupled to the reduction of ferredoxin ($E^{\circ\prime}$ –420 mV). The subsequent oxidation of 2 FADH to 2 FAD (FADH/FAD, $E^{\circ\prime}$ –90 mV) is then coupled to the reduction of a compound with a more positive redox potential ($E^{\circ\prime}$ near 0 V; in the case discussed here, crotonyl-CoA is reduced to butyryl-CoA), thus driving the overall reaction. This behavior is in line with the known disproportionation of the semiquinone $FADH^*$ radical to oxidized FAD and reduced $FADH_2$. The $FADH^*$ semiquinone represents 2% of the flavin nucleotide species at equilibrium at neutral pH (54). The new principle of electron bifurcation is also applicable to autotrophs, as we will see; it is probably widespread and may provide an explanation for various unaccountable findings in different fields of anaerobic metabolism.

## Ferredoxin Reduction by $H_2$: Electron Bifurcation by Soluble [NiFe]-Hydrogenase Plus Heterodisulfide Reductase

We noticed that in *C. kluyveri* electron bifurcation the oxidation of 2 FADH is coupled to the reduction of crotonyl-CoA. Some methanogens (those without cytochromes) energetically couple the first (endergonic) and last (exergonic) steps in methanogenesis from $CO_2$ by a soluble enzyme complex that mediates a flavin-based electron bifurcation (22, 60, 104). In this process the cytoplasmic [NiFe]-hydrogenase (MvhADG) couples the oxidation of $H_2$ to the reduction of the cytoplasmic heterodisulfide reductase (HdrABC), with which it forms a tight soluble complex (**Figure 5*b***).

$$H_2 + HdrABC_{ox} \rightarrow HdrABC^{2-}_{red} + 2\,H^+.$$

The MvhADG/HdrABC complex catalyzes the reduction of the heterodisulfide of coenzyme M (HS-CoM) and coenzyme B (HS-CoB), CoM-S-S-CoB ($E^{\circ\prime}$ –140 mV), with $H_2$ ($E^{\circ\prime}$ –414 mV), yet only at a low rate. Surprisingly, the heterodisulfide-dependent reduction of clostridial ferredoxin with $H_2$ proceeds at a high rate. The complex thus appears to couple the endergonic reduction of ferredoxin ($E' \approx -500$ mV) with $H_2$ to the exergonic reduction of the heterodisulfide with $H_2$. This coupling probably involves the FAD in the subunit HdrA as the center of electron bifurcation. Assuming that the reaction is analogous to the ferredoxin-dependent crotonyl-CoA reduction with NADH, the stoichiometry would be

$$2\,H_2 + Fd_{ox} + CoM - S - S - CoB \rightarrow Fd^{2-}_{red} + 2\,H^+ + CoM - SH + CoB - SH.$$

Hydrogen can be substituted by formate (22). Reduced ferredoxin then serves for all ferredoxin-dependent steps in carbon fixation. In *Methanothermobacter* spp. this ferredoxin is probably a 12[4Fe-4S] polyferredoxin MvhB (103).

## Ferredoxin Reduction by $H_2$: Membrane-Bound Energy-Converting [NiFe]-Hydrogenases

Methanogens with cytochromes have considerably higher growth yields and threshold concentrations for $H_2$ than do methanogens lacking cytochromes. They couple the first and last steps in methanogenesis from $CO_2$ chemiosmotically by energy-converting membrane-bound [NiFe]-hydrogenases, a unique subfamily of the [NiFe]-hydrogenases found in many Eubacteria and Archaebacteria (103). The six conserved subunits share sequence similarity with subunits of formate dehydrogenase-associated [NiFe]-hydrogenase from *Escherichia coli*, of energy-conserving NADH:quinone oxidoreductase (NuoA-N) from *E. coli*, and of carbon monoxide dehydrogenase-associated [NiFe]-hydrogenase from Eubacteria.

In heterotrophs (e.g., *Pyrococcus furiosus*), the enzyme functions in hydrogen production with reduced ferredoxins or polyferredoxins that are

generated in metabolism (e.g., by oxidizing low-potential substrates such as pyruvate or CO) (85). This exergonic reaction is coupled to energy conservation by means of proton (or sodium ion) translocation to the outside (and is therefore energy converting).

$$Fd^{2-}_{red} + 2\,H^+ \rightarrow Fd_{ox} + H_2 + \Delta\mu H^+/Na^+.$$

In chemolithotrophic (autotrophic) $H_2$ consumers, the system functions in vivo in the reverse direction, accomplishing the full reduction of ferredoxin (40, 103) (**Figure 5*c***).

$$Fd_{ox} + H_2 + \Delta\mu H^+/Na^+ \rightarrow Fd^{2-}_{red} + 2\,H^+.$$

In the different lineages of methanogens, various types of those membrane-bound energy-converting [NiFe]-hydrogenases contain six conserved core subunits and up to 14 additional subunits.

## Ferredoxin Reduction by $H_2$: Soluble Bifurcating [FeFe]-Hydrogenases

Another fascinating example of bifurcation, which in heterotrophs such as *Thermotoga maritima* operates in the direction of hydrogen formation, is used by a new class of bifurcating [FeFe]-hydrogenase, catalyzing

$$NADH + Fd^{2-}_{red} + 3\,H^+ \rightarrow NAD^+ + Fd_{ox} + 2\,H_2.$$

Almost one-third of the known $H_2$-producing anaerobes appear to contain homologs of this trimeric bifurcating cytoplasmic enzyme (92); note that the monomeric (FeFe) enzymes directly reduce ferredoxin. It may well be that this enzyme reaction, in the reverse direction, constitutes another example of ferredoxin reduction by hydrogen via a bifurcation mechanism (**Figure 5*d***):

$$2\,H_2 + NAD^+ + Fd_{ox} \rightarrow NADH + Fd^{2-}_{red} + 3\,H^+.$$

## GLUCONEOGENESIS FROM ACETYL-CoA

All anaerobic autotrophic pathways lead to acetyl-CoA. The biosynthesis of $C_3$- to $C_6$-compounds therefore has to spring from acetyl-CoA (**Figure 6*a***). In strict anaerobes, heterotrophs as well as autotrophs, ferredoxin-dependent pyruvate synthase catalyzes the reductive carboxylation of acetyl-CoA, and pyruvate conversion to PEP uses pyruvate:water dikinase or pyruvate:phosphate dikinase. Oxaloacetate arises from carboxylation of pyruvate or PEP, and 2-oxoglutarate is formed by an incomplete reductive citric acid cycle including 2-oxoglutarate synthase or by a horseshoe-type incomplete citric acid cycle lacking 2-oxoglutarate-oxidizing enzyme. Gluconeogenesis starting from PEP appears to be uniform (83) and uses a fructose-1,6-bisphosphate (FBP) aldolase. Interestingly, only a small group of Archaebacteria contains a proven archaebacterial FBP aldolase (94). The majority contain a bifunctional enzyme that shows FBP aldolase and FBP phosphatase activity (37, 87).

This pacemaking enzyme catalyzes the conversion of two triosephosphate molecules directly to fructose-6-phosphate and inorganic phosphate. Interestingly, FBP aldolase/phosphatase is also present in the deep-branching, mostly thermophilic lineages of the Eubacteria, whereas it is missing in most other Eubacteria and in Eukaryota. The highly conserved, heat-stabile, and bifunctional FBP aldolase/phosphatase may represent the ancestral gluconeogenic enzyme. The distribution pattern and the unidirectional catalysis of FBP aldolase/phosphatase suggest that the Embden-Meyerhof-Parnas pathway evolved first in the direction of gluconeogenesis. Combination of the bifunctional aldolase/phosphatase with the modified Entner-Doudoroff pathways in Archaebacteria (95) may allow simultaneous use of different growth substrates, regardless of whether usage of the substrates requires glycolysis (sugar substrates) or gluconeogenesis (other substrates); this hypothesis needs to be tested. Also, heat-labile triosephosphates that decay into toxic methylglyoxal are quickly removed and trapped in stable fructose-6-phosphate. No molecules are left behind (96). Even the toxic methylglyoxal may be used in a novel archaebacterial aromatic biosynthetic pathway (113). The prebiotic biosynthesis of

pentosephosphates and purines and pyrimidines and their nucleotides is especially challenging because these processes are central for the emergence of an RNA world (75).

## RATIONAL FOR DIVERSITY: ANAEROBIC VERSUS AEROBIC METABOLISM AND OTHER FACTORS

To which environment do individual pathways fit best, and did the genes for essential enzymes preexist or were they acquired by lateral gene transfer? Balancing the different outside and inherent necessities of the autotrophic pathways may be what determines, in the present and in the past, whether an autotrophic organism can successfully compete.

### Oxygen

The most decisive factor is the availability of oxygen. Aerobic growth demands oxygen-insensitive enzymes and therefore oxygen-sensitive mechanisms of strict anaerobes cannot

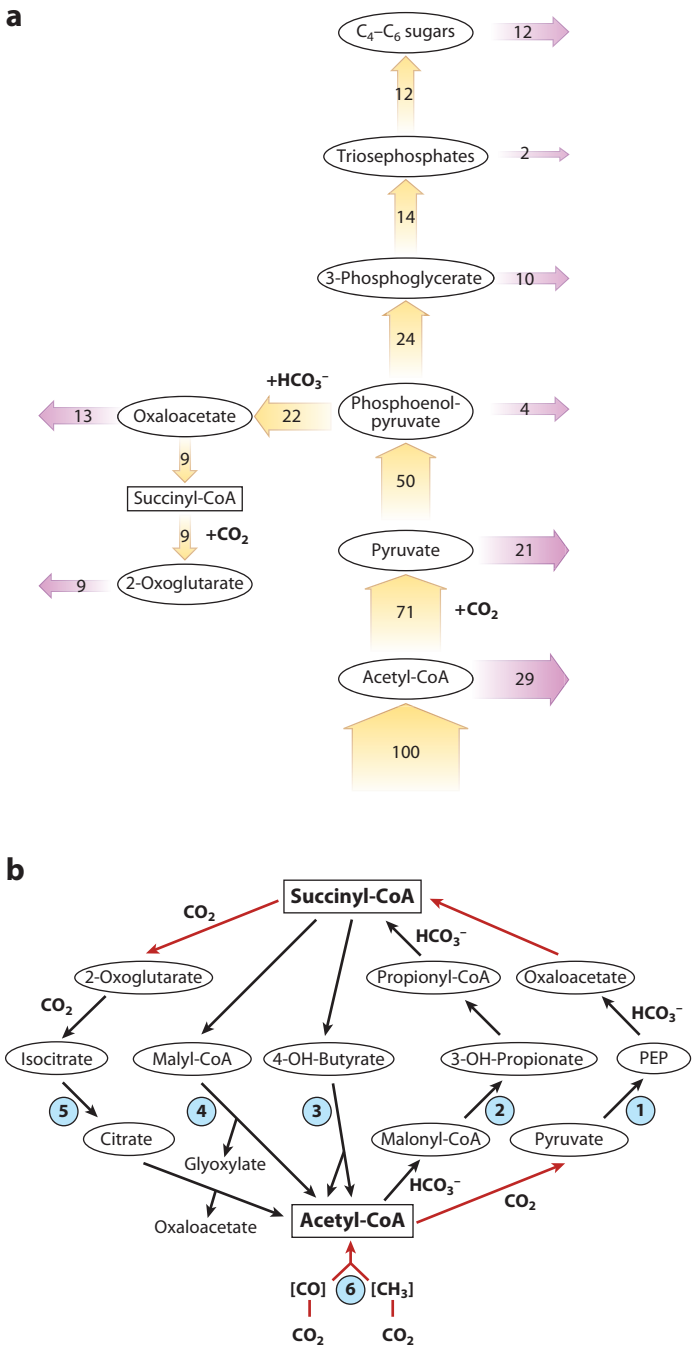



**Figure 6**

(*a*) Ideal blueprint of the primordial anaerobic central carbon metabolism. The numbers inside the arrows indicate the percentage of metabolic fluxes, when all metabolic fluxes of anabolism are taken as 100%. Yellow arrows indicate central metabolism; purple arrows indicate biosynthetic routes departing from the central intermediates. (*b*) Addition of only a few steps indicated in Routes 4, 5, and 6 to the basic route (Route 1) leads to various autotrophic $CO_2$ fixation pathways. The combination of Route 1 and Route 5 yields the reductive citric acid cycle. The combination of Route 1 and Route 3 yields the dicarboxylate/4-hydroxybutyrate cycle. The aerobic routes arose later by combining aerobic Route 2 with Route 3 to yield the 3-hydroxypropionate/4-hydroxybutyrate cycle, and combining Route 2 and Route 4 to yield the first cycle of the 3-hydroxypropionate bi-cycle, which requires an additional cycle for glyoxylate assimilation. Route 6 represents the reductive acetyl-CoA pathway, which requires the Route 1 for the assimilation of acetyl-CoA. Red arrows indicate oxygen-sensitive enzymes/reactions. Route numbers are encircled.

Supplemental Material



function, even if they were more cost-effective. It is safe to assume that the strict anaerobic pathways evolved first and that by implication the oxygen-tolerant pathways evolved later as an adaptation to the new aerobic lifestyle. The three anaerobic pathways have in common the reductive conversion of acetyl-CoA to succinyl-CoA, which requires oxygen-sensitive reduced ferredoxin and 2-oxoacid synthases catalyzing the reductive carboxylation of acyl-CoAs to 2-oxoacids (**Figure 6*b***). This route seems to be a common heritage and a feature of primordial anoxic metabolism. If so, mechanisms designed to keep ferredoxin reduced must be ancestral.

The adaptation to a secondary aerobic lifestyle may be reconstructed in autotrophic Crenarchaeota. The oxygen-sensitive dicarboxylate/4-hydroxybutyrate cycle appears to be a common heritage of Crenarchaeota. The aerobic *Sulfolobales* as well as the aerobic autotrophic marine and soil Crenarchaeota developed another oxygen-insensitive strategy of making succinyl-CoA from acetyl-CoA. They acquired, possibly independently from each other, the 3-hydroxypropionate part of their carbon fixation cycle by using biotin-dependent carboxylases instead of oxygen-sensitive 2-oxoacid synthases. The 4-hydroxybutyrate part of the cycle apparently was adequately oxygen tolerant, even though 4-hydroxybutyryl-CoA dehydratase contains an oxygen-labile iron-sulfur center.

The Calvin-Benson cycle is a special case. It uses oxygen-insensitive enzymes and thus allows for the shift from an anaerobic to an aerobic lifestyle; yet it suffers from the oxygenase side reaction of its key carboxylase. One may assume that during evolution the oxygenase side reaction of RuBisCO was suppressed to a tolerable extent to cope with increasing $O_2$ and decreasing $CO_2$ levels; this adaptation may explain the cycle's success in extant biology (99, 101). Furthermore, the early atmosphere contained probably much more $CO_2$ than the contemporary atmosphere and the low affinity of RuBisCO for $CO_2$ may not have been a disadvantage then.

## Energy Demands

The pathways require different amounts of ATP to make the cellular precursor metabolites. In **Supplemental Table 1** the energy needs are standardized to the formation of pyruvate. Note, however, that the full reduction of ferredoxin normally requires additional energy. Those pathways that are closest to the thermodynamic equilibrium have the lowest average redox potential. Energy limitation exerts a strong selection pressure in favor of energy-saving mechanisms. Clearly, the most energy-limited anaerobes use the least costly mechanisms. The average standard redox potential of the redox reactions of the reductive acetyl-CoA pathway in methanogenic Archaebacteria is –430 mV and in acetogenic Eubacteria it is –340 mV, whereas in the reductive citric acid cycle it is –250 mV (105).

However, the costs for synthesizing all auxiliary, $CO_2$-fixation-related enzymes actually might determine decisively the energy costs. The synthesis of the catalysts itself may devour a huge amount of energy as well as nitrogen and sulfur sources, especially if the pathways are long, requiring many auxiliary enzymes. Carboxylases with low catalytic efficiency need to be synthesized in large amounts, as is the case for RuBisCO. To evaluate the costs of a pathway, one must know the exact amount of additional protein required to turn a heterotroph into an autotroph. These costs depend on the number and size of additional enzymes and their catalytic efficiency. In this respect, the Calvin-Benson cycle with only two additional enzymes may be quite cost-effective, especially under high ancestral $CO_2$ concentrations.

## Coassimilation of Organic Compounds

Many autotrophic Eubacteria and Archaebacteria living in aquatic habitats probably encounter carbon oligotrophic conditions and may grow as mixotrophs. Coassimilation of traces of organic compounds may pay off. Some substrates that are more reduced than the average cell carbon require $CO_2$ fixation as a means to consume

excess reducing equivalents that arise during assimilation under anoxic conditions.

The reductive acetyl-CoA pathway is ideally suited for coassimilation of all kinds of one-carbon compounds and acetate (**Figure 1**). Additional cobalamin-dependent methyltransferases react with a wide range of natural methyl donors by activating the methyl group that will be transferred by donating positive charge to the heteroatom (O, N, or S) attached to the methyl group. They feed these one-carbon units into the acetyl-CoA pathway by a methyltransferase system composed of methyl transferase I, which transfers the methyl group from the donor to the corrinoid (protein), from which it is transferred to the acceptor coenzyme by methyl transferase II (78).

The singular development of the 3-hydroxypropionate bi-cycle in *Chloroflexi* was fostered most likely in an aerobic environment, in which traces of various organic products could be coassimilated (118). However, the low amount of available organic substrates may not cope with the potential growth rate of this phototrophic bacterium, thus necessitating additional $CO_2$ fixation, a process that also traps excess reducing equivalents. A complete or even a rudimentary 3-hydroxypropionate/4-hydroxybutyrate cycle or 3-hydroxypropionate bi-cycle allows coassimilation of hosts of organic compounds, which are excreted by other microorganisms or algae, such as the abundant osmoprotectant dimethylsulfoniopropionate, which yields 3-hydroxypropionate (106) (**Figures 3** and **4**). Various widespread marine aerobic (mostly phototrophic) bacteria appear to have acquired genes of a rudimentary 3-hydroxypropionate bi-cycle possibly for that purpose (118). Similarly, the dicarboxylate/4-hydroxybutyrate cycle allows for coassimilating organic substrates (81), whereas the Calvin-Benson cycle is least suited to this purpose.

## Connection with Energy Metabolism and Reversibility

The reductive acetyl-CoA pathway in methanogens and acetogens has a dual function. It is mainly part of their energy metabolism, and anabolism and catabolism are intimately interconnected. Thus, the usage of this pathway for biosynthesis is predestined, as they need much of the one-carbon transforming machinery anyhow. The formation of acetyl-CoA in methanogens requires only two more enzymes (four proteins), methyl transferase plus a methyl-accepting corrinoid protein and CO dehydrogenase/acetyl-CoA synthase. As pointed out, the free energy change associated with the synthesis of acetyl-CoA is the lowest of all pathways. The reductive acetyl-CoA pathway, as well as the reductive citric acid cycle, operates near the thermodynamic equilibrium and can be reversed to oxidize acetyl-CoA (88, 89, 105), if needed. All other autotrophic pathways are unidirectional.

## Metabolic Fluxes

Prokaryotes do not contain masses of lignocellulose-containing cell walls as plants do. Not only cellulose biosynthesis, but also lignin biosynthesis starts from sugar phosphates (erythrose 4-phosphate and PEP). The need for huge amounts of sugars in plants may favor the Calvin-Benson cycle, which turns around sugar phosphates. Prokaryotes, however, need far less sugar phosphates. Rather, their metabolic fluxes are diverted mainly from acetyl-CoA (29%), pyruvate (21%), oxaloacetate (13.5%), and 2-oxoglutarate (8.3%) (**Figure 6*a***). In Archaebacteria all methanogens (except *Methanosarcinales*) as well as *Desulfuroccales* synthesize 2-oxoglutarate via succinyl-CoA rather than via citrate. This strategy is also found in various strict anaerobic Eubacteria. Only 25% of the metabolic fluxes start from intermediates beyond PEP and 14% beyond 3-phosphoglycerate. The required amounts of these central precursor metabolites follow exactly the order in which these metabolites are produced in the central carbon metabolism of most anaerobes. This is the most economical order one could conceive and represents a kind of "ontogeny recapitulates phylogeny" example: Present day metabolic fluxes mirror the prebiotic events, leading from acetyl-CoA via pyruvate, then oxaloacetate,

to 2-oxoglutarate and from pyruvate/PEP to sugar phosphates.

Therefore, autotrophic pathways directly yielding acetyl-CoA are optimal in terms of simplicity of operation. Furthermore, in case of the Calvin-Benson cycle the synthesis of a central precursor metabolite, acetyl-CoA, from 3-phosphoglycerate is connected with the loss of $CO_2$. Yet, most (facultative) aerobic Eubacteria use the Calvin-Benson cycle, whose regulation is almost detached from the central carbon metabolism, rendering its control particularly robust. Robustness of regulation of a pathway may be crucial, but an autotrophic pathway's suitability for simultaneous or consecutive utilization of organic compounds may be as important.

## $CO_2$ Species

The concentration of bicarbonate in slightly alkaline water is much higher than the concentration of dissolved $CO_2$ (apparent $pK_a$ $[HCO_3^-/CO_2] = 6.3$); autotrophs therefore may profit from using bicarbonate instead of $CO_2$. The $CO_2$ concentration in water equilibrated with air (pH 7.4, 20°C) is 0.012 mM, whereas that of bicarbonate is 0.26 mM (seawater has a pH value between 7.5 and 8.4). The usage of bicarbonate is a special feature of PEP carboxylase and biotin-dependent carboxylases (e.g., acetyl-CoA/propionyl-CoA carboxylase, pyruvate carboxylase), and the much higher bicarbonate concentration might make up for a possibly lower bicarbonate affinity of the enzymes.

# EVOLUTIONARY CONSIDERATIONS

The common ancestor of life was probably a chemolithoautotrophic thermophilic anaerobe (69, 109, 110). Therefore, extant chemolithoautotrophic thermophilic anaerobes that make their living simply on volcanic gases may provide the best model for primordial metabolism (assuming their lifestyle is primordial). What is their common blueprint?

## Primitive Blueprint of Carbon Metabolism

The main fixed type of metabolism is 12 central precursor metabolites that can be connected by different lines. Reactions that are mechanistically not too demanding tend to lead to multiple solutions, as seen here. Vice versa, mechanistically demanding enzymes are normally conserved. All anaerobic autotrophic pathways lead first to acetyl-CoA. No matter how acetyl-CoA is made from $CO_2$, its further conversion to the other central precursor metabolites is remarkably uniform (83, 96) (**Figure 6*a***). All anaerobic strategies use ferredoxin-dependent pyruvate synthase to make pyruvate and normally use PEP carboxylase to make oxaloacetate. Many anaerobes use reactions of a complete or incomplete reductive citric acid cycle, by which oxaloacetate is reduced to succinyl-CoA or even further to 2-oxoglutarate (33). Furthermore, gluconeogenesis from PEP is similar, and a bifunctional fructose bisphosphate aldolase/phosphatase renders hexosephosphate synthesis irreversible in Archaebacteria and in early lineages of Eubacteria (87). This central metabolic network described in **Figure 6*a*** may be considered basic. It requires sophisticated mechanisms to keep ferredoxin reduced. Which of the anaerobic autotrophic pathways is the best candidate to provide acetyl-CoA? Given the basic blueprint, it requires not much to complete the scheme in **Figure 6*a*** to an autotrophic reductive citric acid cycle or to a dicarboxylate/4-hydroxybutyrate cycle by adding only a few autotrophy-specific enzyme steps (**Figure 6*b***). Yet, several aspects of the reductive acetyl-CoA pathway are unique and intuitively this pathway might be considered closer to ancestral autotrophic carbon fixation (14, 32).

## Use of Volcanic Gases and Propinquity to Chemical Processes

This pathway uses CO as an intermediate; CO is a common volcanic gas with strong reduction potential enabling reduction of ferredoxin

up to the level of –500 mV. CO feeds into the pathway by spontaneously reacting with CO dehydrogenase/acetyl-CoA synthase. This enzyme probably has common roots in Eubacteria and Archaebacteria (77), which admittedly may be indicative of a metabolic trait rather than a phylogenetic trait. Similarly, formaldehyde spontaneously adds to tetrahydropterin, forming 5,10-methylene-tetrahydropterin. In addition to CO and formaldehyde, prebiotic inorganic carbon could also include COS, HCN, $CH_3SH$, and other one-carbon molecules that are partly reduced and more reactive than $CO_2$. Note that formate, methanol, methylamine, methylmercaptane, and acetate can feed into the pathway without great effort. Hence this pathway is ideally suited for assimilating one-carbon units on a variety of oxidation levels, especially volatile compounds present in volcanic exhalations. The reductive acetyl-CoA pathway can be considered a biological equivalent of the industrial Monsanto process, by which acetate is produced from CO and methanol through metal catalysis. It can also be considered a kind of biological Fischer-Tropsch process, in which CO is built up to multicarbon compounds.

## Minimal Energy Requirements and Linkage to Energy Metabolism

The reductive acetyl-CoA pathway has minimal energy requirements and intimately links anabolism and catabolism, because the product of the pathway, acetyl-CoA, can be converted to acetate under formation of ATP. A prerequisite of the functioning of a metabolic cycle is its linkage to any kind of energy-providing process, and this pathway features this coupling. The reductive acetyl-CoA pathway is not restricted to methanogenic Archaebacteria but occurs in several strictly anoxic groups of the Eubacteria and can be reversed for the oxidation of acetyl-CoA, depending on the growth substrates (88). How ferredoxin is fully reduced and how an energized membrane is built are central questions to the evolution of primordial metabolism (64, 73).

## Role of Metals and Coenzymes

The reductive acetyl-CoA pathway makes extensive use of coenzymes (tetrahydropterin, cobalamin and others, depending on the systematic position of the organism), metals, and Fe-S or Fe-Ni-S centers with CO and $CN^-$ ligands. Coenzymes and metal centers probably preceded the more complex proteins as catalysts. The reduced forms of the relevant transition metals (V, Mn, Fe, Co, Ni, Cu, Zn, Mo, W) are generally more soluble than the oxidized forms that are formed under oxic conditions. The process involves the stepwise formation of organometallic intermediates (methyl-Co, Ni-CO, methyl-Ni, acetyl-Ni) (78). This pathway never adapted to oxic conditions because it uses low-potential electron donors, and some of its enzymes, notably CO dehydrogenase/acetyl-CoA synthase, are inherently highly oxygen sensitive.

## In Vitro Simulation

Inorganic carbon fixation may have proceeded on minerals and may have been based on catalysis by transition metal sulfides. Given the structural (and catalytic) similarity between the minerals themselves and the catalytic metal or Fe-S-containing centers of the enzymes or cofactors in the acetyl-CoA pathway, one attractive idea is that minerals catalyzed a primitive acetyl-CoA pathway (84, 110). Indeed, the presumed primordial situation can be mimicked successfully in vitro. For example, both the thioester acetyl methylsulfide and its hydrolyzed product, acetate, can be produced from CO and $CH_3SH$ using only Fe- and Ni-sulfides as catalysts. Even higher molecular compounds can be synthesized (47).

# HILBERT'S LIST OF AUTOTROPHY ISSUES

Although six autotrophic pathways seem to be enough, this case is probably not the last word on autotrophy. One can design many other synthetic pathways on paper (10), and

there are possibly more autotrophic pathways out there. In addition to watching out for new cycles beginning with genome searching, several issues related to the known pathways deserve to be addressed (see Future Issues, below).

## SUMMARY POINTS

1. Five alternative pathways of carbon fixation that exist in addition the Calvin-Benson cycle are reviewed.
2. The energetics of the reactions of the pathways are estimated and the mechanisms of how reduced ferredoxin can drive low-potential reduction reactions are discussed.
3. A rationale for the superiority of the individual pathways under natural conditions is presented.
4. A common blueprint of primordial biosynthesis is derived from the common features of the known autotrophic pathways found in anaerobic Eubacteria and Archaebacteria. This blueprint has an impact on theories of the evolution of metabolism.
5. A list of unsolved autotrophy-related issues is compiled.

## FUTURE ISSUES

1. The amount of extra enzyme protein required to turn a heterotroph into an autotroph needs to be evaluated in model organisms, optimally using $H_2$ as reductant. This amount determines how much an autotrophic pathway costs, in addition to the energy required to build up a dozen central metabolites from $CO_2$.
2. The mechanisms by which anaerobes keep ferredoxin strongly reduced need to be identified. The problem is also related to energy coupling in acetogenic bacteria in the course of acetyl-CoA formation from 2 $CO_2$ and 4 $H_2$.
3. The phylogeny of individual key enzymes of autotrophic pathways needs to be studied in order to trace the origin of the pathways. The autotrophic pathway to be elucidated in marine Crenarchaeota may provide insight into convergent evolution in this deep-branching lineage and its possible thermophilic origin.
4. The role of RuBisCO in Archaebacteria is disputable, even though some answers have been given.
5. Some prokaryotes can use one and the same pathway either for making acetyl-CoA from $CO_2$ or for oxidizing acetyl-CoA to $CO_2$; what determines which direction prokaryotes will go? Organisms with more than one autotrophic pathway may exist; which factors govern their usage?
6. Converting a heterotroph to an autotroph is a plausible goal of synthetic biology; it will teach us regulation and will reveal what else is needed to fit an engineered pathway into an existing metabolic network.

7. Further laboratory experiments mimicking syntheses of organic compounds from volcanic gases with metal ion catalysis under high temperature are required. The microbial assimilation of other volcanic gases such as HCN, COS, and $CH_3SH$, which contain carbon and are secondarily formed from the primary gases, might result in new findings.
8. Conceptually, the coevolution of the basic ingredients of life that are necessary to form a functioning and viable life unity is a challenge that has a great impact on our weltanschauung. How are biosynthesis, energy metabolism, energized membrane structure, and primitive hereditary mechanism (the RNA world) logically linked?

## DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

## ACKNOWLEDGMENTS

This work was supported by Deutsche Forschungsgemeinschaft and Evonik-Degussa. I thank Ivan Berg for invaluable help with the preparation of the manuscript and for stimulating discussions and Rudolf Thauer and Wolfgang Buckel for disputing mechanisms of ferredoxin reduction. I thankfully acknowledge the contributions of all coworkers and cooperation partners over the past 35 years.

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Volume 65, 2011

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**Errata**

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