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# Electron Bifurcation: A Long-Hidden Energy-Coupling Mechanism

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## Abstract

A decade ago, a novel mechanism to drive thermodynamically unfavorable redox reactions was discovered that is used in prokaryotes to drive endergonic electron transfer reactions by a direct coupling to an exergonic redox reaction in one soluble enzyme complex. This process is referred to as flavin-based electron bifurcation, or FBEB. An important function of FBEB is that it allows the generation of reduced low-potential ferredoxin ( $Fd_{red}$ ) from comparably high-potential electron donors such as NADH or molecular hydrogen ( $H_2$ ).  $Fd_{red}$  is then the electron donor for anaerobic respiratory chains leading to the synthesis of ATP. In many metabolic scenarios,  $Fd$  is reduced by metabolic oxidoreductases and  $Fd_{red}$  then drives endergonic metabolic reactions such as  $H_2$  production by the reverse, electron conformation. FBEB is energetically more economical than ATP hydrolysis or reverse electron transport as a driving force for endergonic redox reactions; thus, it does “save” cellular ATP. It is essential for autotrophic growth at the origin of life and also allows for heterotrophic growth on certain low-energy substrates.



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## INTRODUCTION

The two energy currencies present in every living cell, ATP and the electrochemical potential of  $H^+$  or  $Na^+$  ( $\Delta\tilde{\mu}_{H^+}$  or  $\Delta\tilde{\mu}_{Na^+}$ ), are used as driving forces for endergonic reactions needed to build biomass and sustain life. A decade ago, a mechanism to drive thermodynamically unfavorable reactions was discovered that is independent of ATP and/or  $\Delta\tilde{\mu}_{H^+}$  or  $\Delta\tilde{\mu}_{Na^+}$  (14, 41). It is used in prokaryotes to drive endergonic electron transfer reactions by a direct coupling to an exergonic redox reaction in one soluble enzyme complex. This mechanism is called electron bifurcation because it splits an electron pair: One goes down the energy gradient and drives the other up. This is not without precedence; electron bifurcation was first discovered in the cytochrome  $b_{c_1}$  complex (13).

Flavins are involved as electron splitters in electron bifurcation, and thus the process is referred to as flavin-based electron bifurcation, or FBEB. FBEB is not a third mechanism of energy conservation—as often stated—because it is not coupled to ATP synthesis (15).

One important function of FBEB is that it allows the generation of reduced low-potential ferredoxin ( $Fd_{red}$ ) with molecular hydrogen ( $H_2$ ) or NADH as reductant.  $Fd_{red}$  is then used as an electron donor for anaerobic respiratory chains. In many heterotrophic pathways, ferredoxin ( $Fd$ ) is the electron acceptor of some oxidoreductases, and  $Fd_{red}$  then drives endergonic metabolic reactions such as  $H_2$  production by the reverse, electron confurcation.



Involvement of FBEB in anaerobic metabolism also saves cellular ATP and is energetically more economical than ATP or  $\Delta\tilde{\mu}_{H+}$  or  $\Delta\tilde{\mu}_{Na+}$  as a driving force for endergonic reactions. This is of particular importance for anaerobes that grow at the thermodynamic limit of life. In these cells, FBEB allows for net ATP synthesis from certain low-energy substrates and thus enables growth on such substrates. This review focuses on the discovery of FBEB, its distribution in bacteria and archaea, and its physiological role and biochemistry.

## DISCOVERY OF FLAVIN-BASED ELECTRON BIFURCATION

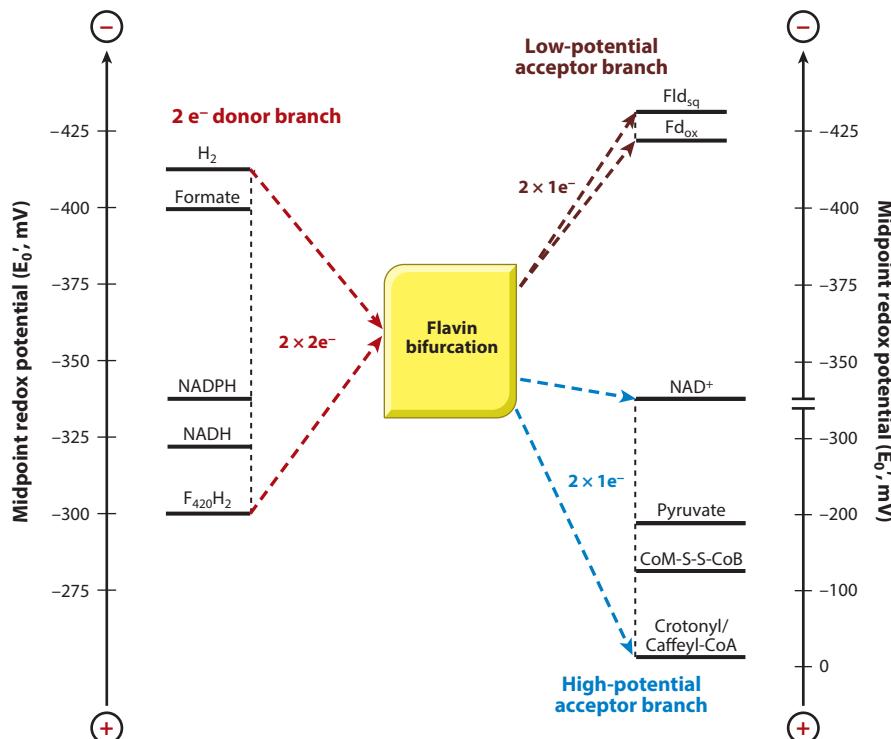
Anaerobic microorganisms that thrive in the absence of electron acceptors live by fermentation, a process in which the organic substrate is first oxidized to an intermediate that is then reduced to an end product (73). Lactate fermentation is the simplest form: Glucose is oxidized to pyruvate plus 2 reducing equivalents (4 electrons), and pyruvate is then reduced to lactate using these 2 reducing equivalents. This balances out the electrons and leaves the cell with 2 mol of ATP generated by substrate-level phosphorylation (SLP). An increase in ATP could be achieved if pyruvate were oxidized to  $CO_2$  and acetyl-CoA and the latter were converted to acetate via phosphotransacetylase and acetate kinase. This would yield 2 additional ATP in the acetate kinase reaction and increase the energy balance of glucose fermentation by 100%. However, it leaves the cells with the problem of expelling the reducing equivalents. The easiest way to do this would be to discard them as  $H_2$ , but here the cell faces a tremendous energetic problem: The redox potential of the NADH/NAD couple is too electropositive ( $E_0' = -320$  mV) to reduce protons ( $E_0' = -414$  mV). Bacteria do produce  $H_2$  from low-potential electron carriers such as Fd ( $E_0' = -410$  mV to  $-500$  mV), but the question of how Fd is reduced with NADH as reductant remained unanswered for decades. Generations of microbiologists grew up with the question of how *Clostridium kluyveri* produces  $H_2$  from ethanol and acetate (99, 100, 103). This organism has gathered immense attention from biotechnologists since it converts acetate and ethanol to butyrate and caproate and the aforementioned  $H_2$  (32, 82, 86). The enigma of how  $H_2$  is formed was solved in 2008 by W. Buckel and R. Thauer, bringing to light a new perspective on energy coupling in anaerobic metabolism (14, 41, 60).

Cell-free extracts of *C. kluyveri* catalyzed the formation of  $H_2$  from NADH in the presence of crotonyl-CoA (60). The authors reporting this speculated that the endergonic reduction of Fd ( $E_0' = -410$  mV) with NADH ( $E_0' = -320$  mV) as reductant is energetically driven by the concomitant exergonic reduction of crotonyl-CoA to butyryl-CoA ( $E_0' = -10$  mV). To test this hypothesis, the crotonyl-CoA reductase/butyryl-CoA dehydrogenase was purified. The enzyme complex catalyzing crotonyl-CoA reduction contained three subunits, the dehydrogenase (Bcd) and the two subunits of an electron-transferring flavoprotein (EtfAB). The enzyme complex catalyzed crotonyl-CoA reduction with NADH, albeit with low activities. Addition of Fd purified from *C. kluyveri* stimulated NADH oxidation 20-fold, and indeed, Fd was reduced [measured as  $H_2$  production catalyzed by hydrogenase (77) from *Clostridium pasteurianum*], according to the following reaction:



The enzyme contained FAD, and FAD was essential for activity as well as for stability. In an accompanying publication, Herrmann et al. (41) put forward a hypothesis for the mechanism of energy coupling in the enzyme complex, with the flavins as essential components for the reaction mechanism. Equally important was their prediction of different electron-bifurcating reactions in other bacteria and archaea and their importance for the energy metabolism of anaerobes in general.



**Figure 1**

Examples of electron bifurcation systems in anaerobes. Horizontal bars along the scale indicate midpoint redox potentials ( $E_0'$  at pH 7.0) of different electron donors and acceptors. Flavin-based electron bifurcation center in yellow; in red, 2-electron transfer from donor; in brown and blue, 1-electron transfer to low-potential and high-potential acceptor, respectively. (Redox potential not drawn to scale.) Abbreviations: CoB, coenzyme B; CoM, coenzyme M; Fdox, oxidized ferredoxin; Fldsq, semiquinone state flavodoxin.

Soon after the first discovery of FBEB in 2008 (41, 60), a number of enzymes were reported that use the mechanism of FBEB to couple two redox reactions (4, 92, 95, 109, 111–113). The electron-bifurcating reactions described to date are summarized in **Figure 1**.

## ELECTRON BIFURCATION INDIRECTLY INCREASES CELLULAR ENERGY LEVELS IN ANAEROBES

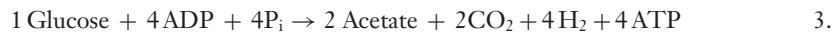
### Hydrogen Production Increases Cellular ATP Yields

Anaerobic microbes fermenting sugars synthesize ATP by the reactions linked to SLP: the phosphoglycerate kinase and pyruvate kinase reactions (73). Redox balancing is achieved by reducing pyruvate to lactate or by reducing acetyl-CoA, derived from the oxidative decarboxylation of pyruvate, to ethanol or to other compounds. However, if NADH were reoxidized with  $H^+$  as electron acceptor, yielding  $H_2$ , acetyl-CoA could be converted to acetyl phosphate and then to acetate, yielding additional ATP by SLP:



Indeed, electron-bifurcating hydrogenases can operate in reverse (95) (confurcating) mode, producing  $H_2$  from NADH (and  $Fd_{red}$ ). Such an enzyme was isolated from the thermophilic bacterium

*Thermotoga maritima*. The use of the electron-bifurcating hydrogenase enables *T. maritima* to conserve 4 ATP from glucose fermentation (Reaction 3), which is based on H<sub>2</sub> production from NADH and Fd<sub>red</sub>, thus putting a new perspective on biological H<sub>2</sub> production.



A similar lifestyle is observed in *Ruminococcus albus* (119). These are prime examples of how electron bifurcation considerably increases cellular ATP yields in an indirect manner.

### Electron Bifurcation Generates a High-Energy Intermediate That Drives Soluble, Endergonic Reactions

Just as ATP is an ideal energy currency that can couple exergonic and endergonic reactions, the same is true for Fd<sub>red</sub>, but this is restricted to redox reactions. Methanogenic archaea as well as acetogenic bacteria grow by reduction of CO<sub>2</sub> to methane and acetate, respectively, with H<sub>2</sub> as reductant (92, 102). In acetogens, CO<sub>2</sub> is first reduced to formate, which is then bound in an energy-dependent and ATP-driven reaction to tetrahydrofolic acid, yielding formyl-tetrahydrofolic acid (48, 62, 84, 85). In methanogens, CO<sub>2</sub> is directly bound to the C<sub>1</sub> carrier methanofuran, giving formyl-methanofuran (11, 59). This reaction requires not ATP but Fd<sub>red</sub> as a driving force (57). This is the first example in this review that ATP and Fd<sub>red</sub> can be energetically equivalent in driving endergonic reactions. The question of how reduction of Fd can be achieved has been an enigma for decades. Cell-free extracts of methanogens reduce CO<sub>2</sub> with H<sub>2</sub> as reductant with very low rates. It was noted by R.P. Gunsalus & R. Wolfe (36) that addition of coenzyme M (CoM) dramatically stimulates CO<sub>2</sub> reduction (the RPG effect). Today, it is clear that Fd<sub>red</sub> is required as a driving force, but again the same thermodynamic problem of reducing Fd with H<sub>2</sub> applies. Methanogens with cytochromes, such as *Methanosarcina* species, have a respiratory chain involving a membrane-bound hydrogenase, methanophenazine, cytochromes, and a heterodisulfide reductase (24, 26, 49). The latter enzyme reduces the heterodisulfide and is the terminal enzyme of a respiratory chain. The respiratory chain is coupled to electrogenic translocation of protons across the cell membrane to establish Δμ<sub>H+</sub> (10, 25). The Δμ<sub>H+</sub> is then used as a driving force for a reverse electron flow from H<sub>2</sub> to Fd, catalyzed by an energy-converting hydrogenase, Ech (114, 115). In cytochrome-free methanogens, electron bifurcation replaces Δμ<sub>H+</sub> as a driving force. There, the electron-bifurcating heterodisulfide reductase directly couples H<sub>2</sub>-dependent heterodisulfide reduction to the reduction of Fd, without the intermediary involvement of Δμ<sub>H+</sub>. This is the first example showing that Δμ<sub>H+</sub> and electron bifurcation are two alternative mechanisms to drive the endergonic reduction of Fd.

### ELECTRON BIFURCATION PROVIDES REDUCED FERREDOXIN THAT DRIVES ION-MOTIVE RESPIRATORY CHAINS

Two classes of Fd-dependent respiratory complexes are known: the Ech hydrogenase (38) and the Rnf complex (8, 50). The former has in its simplest form six subunits. EchA and EchB are membrane integral and couple electron transfer from Fd<sub>red</sub> to protons, thereby producing H<sub>2</sub>. Electron carriers involved are FeS clusters and flavins. The potential difference between Fd<sub>red</sub> and protons is used for electrogenic and endergonic export of Na<sup>+</sup> or H<sup>+</sup> to establish a Δμ<sub>H+</sub> or Δμ<sub>Na+</sub> across the cytoplasmic membrane. Although there is quite a bit of circumstantial evidence for this notion, however, it should be noted that a final proof awaits the purification and reconstitution of the enzyme complex into liposomes. More complex hydrogenases of this type are the membrane-bound hydrogenases present in archaea such as *Pyrococcus* species (53, 67) and



*Thermococcus* species (54) that are also adapted to use other reductants such as formate or CO and have potential  $\text{Na}^+/\text{H}^+$  antiporter modules to exchange  $\Delta\tilde{\mu}_{\text{H}^+}$  and  $\Delta\tilde{\mu}_{\text{Na}^+}$  (75, 118).

The second Fd-dependent respiratory enzyme found in anaerobes, both bacteria and archaea, is the Rnf complex (6, 8, 9, 44, 90, 104). A decade ago, a membrane-bound Fd:NAD<sup>+</sup> oxidoreductase activity was discovered in the acetogenic bacterium *Acetobacterium woodii* (50). Three years later, it was demonstrated that this activity is coupled to a vectorial  $\text{Na}^+$  transport across the cytoplasmic membrane (6). This novel type of respiratory enzyme was enriched from *A. woodii* and shown to be encoded by the *rnf* (*Rhodobacter* nitrogen fixation) genes (9). Homologs of these genes were found in the purple nonsulfur bacterium *Rhodobacter capsulatus* and, by mutational analysis, shown to be involved in nitrogen fixation (91). The *rnf* genes are widespread in bacteria and are also found in some archaea (8). Some Rnf complexes use  $\text{Na}^+$  as a coupling ion, and others use protons (42). Mutational analyses in *Methanosaerina acetivorans* (90), *Bacteroides fragilis* (42), and *Clostridium ljungdahlii* (104) proved that the ion-translocating Fd<sub>red</sub>:NAD<sup>+</sup> oxidoreductase activity is indeed encoded by the *rnf* genes.

The membrane-integral Rnf complex contains, in its simplest form, six subunits. It has FeS clusters as well as flavins as electron carriers. In contrast to Ech, the electron acceptor here is not a proton but NAD<sup>+</sup>. Thus, the difference in redox potential is greater compared to Ech, and this is why Ech complexes are suggested to pump 1 proton per 2 electrons whereas Rnf may pump 1 proton per 1 electron.

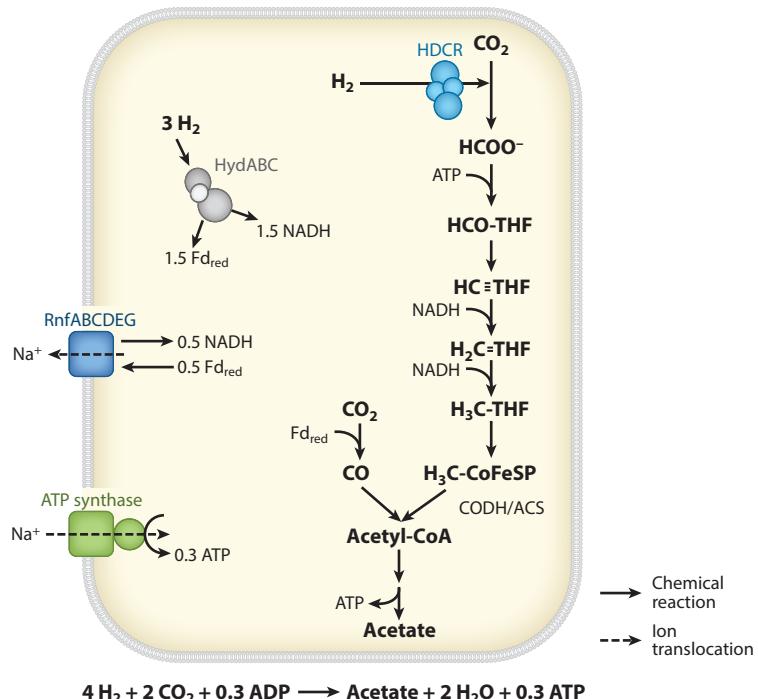
## ELECTRON BIFURCATION ALLOWS GROWTH ON LOW-ENERGY SUBSTRATES

Acetogenesis from  $\text{H}_2 + \text{CO}_2$  according to



as carried out by a specialized group of bacteria, the acetogenic bacteria, is clearly at the thermodynamic limit of life. Taking into account the  $\text{H}_2$  partial pressures that have been determined in anaerobic ecosystems (34), the free energy change of this reaction is around  $-20 \text{ kJ/mol}$ , just enough to synthesize 0.3 ATP, considering a physiological phosphorylation potential of around  $60 \text{ kJ/mol}$  (75). Recently, the phosphorylation potential in *A. woodii* has been determined to be around  $30 \text{ kJ/mol}$ , depending on the substrate (97). This low phosphorylation potential helps to sustain life under these conditions, but even so only 0.6 ATP can be made from the reaction (Equation 4). How energy is conserved during acetogenesis has been solved in the model acetogen *A. woodii*. This acetogen employs an electron transport chain that is disparate from the pathway of acetogenesis and contains an Rnf complex and an ATP synthase, connected by a sodium ion circuit (94). The respiratory enzyme, the Rnf complex, is fueled with Fd<sub>red</sub>, and NADH is the end product of the respiration. NADH is reoxidized by reducing  $\text{CO}_2$  to acetate; thus, the  $\text{CO}_2$  reduction pathway can be regarded as a sink for electrons (as well as a pathway for carbon fixation). Fd reduction with  $\text{H}_2$  as reductant is only possible by employing an electron-bifurcating hydrogenase that generates NADH and Fd<sub>red</sub> in equal amounts. The  $\text{CO}_2$  reduction pathway in *A. woodii* (Wood-Ljungdahl pathway, WLP) requires 4 reducing equivalents, 2 in the form of 2 NADH, 1 in the form of Fd<sub>red</sub>, and 1 in the form of  $\text{H}_2$ , for the first step in  $\text{CO}_2$  fixation, the  $\text{H}_2$ -dependent  $\text{CO}_2$  reductase (93). The electron-bifurcating hydrogenase oxidizes 3 mol of  $\text{H}_2$ , yielding 1.5 mol NADH and 1.5 mol Fd<sub>red</sub>. One mole of Fd<sub>red</sub> is used for the CO dehydrogenase reaction, which leaves only 0.5 mol of Fd<sub>red</sub> for the Rnf complex, allowing for the translocation of 1 mol of  $\text{Na}^+$ . The ATP synthase has a stoichiometry of  $3.66 \text{ Na}^+/\text{ATP}$  (12, 31, 64); thus, 0.27 ATP can be made according to Equation 4 (Figure 2). Evidently, electron bifurcation is





**Figure 2**

Acetogenesis from  $\text{H}_2 + \text{CO}_2$  as carried out by *Acetobacterium woodii*.  $\text{H}_2$  is oxidized by an electron-bifurcating hydrogenase (HydABC), which reduces  $\text{Fd}$  and  $\text{NAD}^+$ .  $\text{CO}_2$  is reduced in the Wood-Ljungdahl pathway. Excess  $\text{Fd}^{2-}$  is oxidized by the Rnf complex, which concomitantly reduces  $\text{NAD}^+$ . This exergonic reaction drives the buildup of a  $\text{Na}^+$  gradient that is exploited by a  $\text{Na}^+$ -dependent ATP synthase. For simplicity, a rounded ATP yield of 0.3 (not 0.27) per acetate is given. Abbreviations: CODH/ACS, carbon monoxide dehydrogenase/acetyl-CoA synthase; CoFeSP, corrinoid-iron-sulfur protein; Fd, ferredoxin; HDCR, hydrogen-dependent carbon dioxide reductase; THF, tetrahydrofolate.

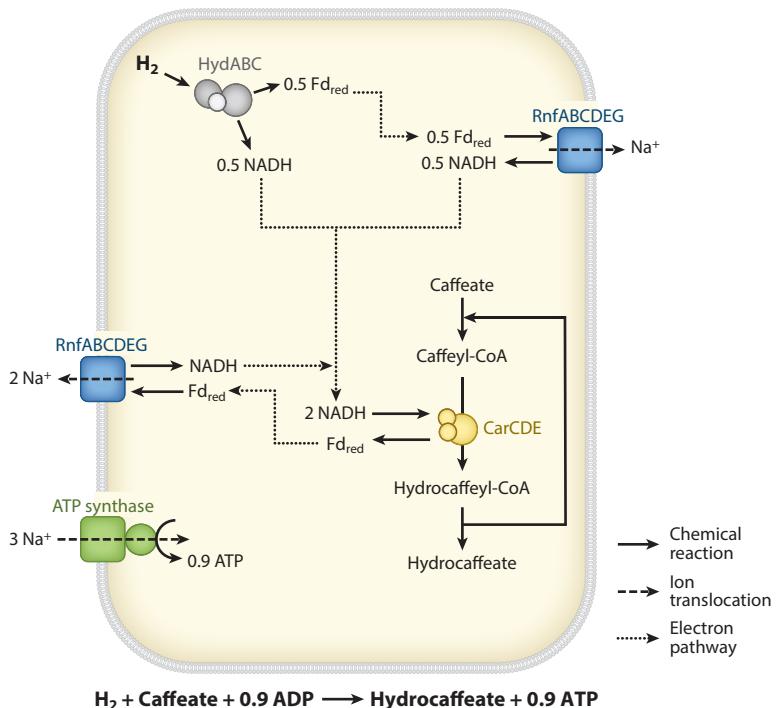
essential for the pathway in reducing  $\text{Fd}$ , the fuel for the Rnf complex. It should be mentioned that a reaction of the carbon pathway, the methylene-THF reductase, may be coupled to  $\text{Fd}$  reduction by electron bifurcation as well. This would supply some additional fuel for the Rnf complex. Yet the enzyme from *A. woodii* does not bifurcate (3), and electron bifurcation was not demonstrated for the enzyme from *Moorella thermoacetica* (70). However, from the inability of the enzyme from *M. thermoacetica* to oxidize  $\text{NADH}$  with methylene-THF as oxidant, it was speculated that a second oxidant is required, as is usually found in electron-bifurcating enzymes.

Caffeate respiration is a type of anaerobic respiration discovered in *A. woodii* (37, 105). This acetogen can grow on a number of other plant-derived compounds and is also able to derive  $O$ -methyl groups from lignin as a carbon and energy source. Additionally, it can use the double bond in compounds such as caffeate as electron acceptors that are reduced to hydrocaffeate according to



The enzymology of this reaction sequence and its bioenergetics was elucidated recently (4, 27, 43, 45, 51) (Figure 3). Caffeate is activated to caffeyl-CoA prior to its reduction. This is achieved initially by an AMP- and pyrophosphate ( $\text{PP}_i$ )-forming caffeyl-CoA synthetase. The  $\text{PP}_i$  is then

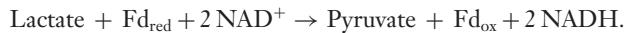


**Figure 3**

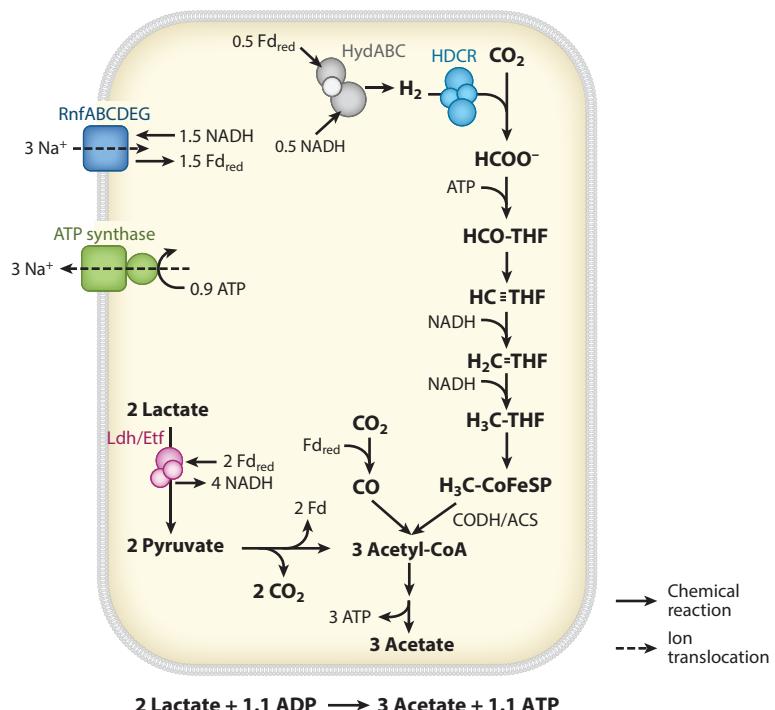
Caffeate respiration as carried out by *Acetobacterium woodii*. H<sub>2</sub> is oxidized by an electron-bifurcating hydrogenase (HydABC), which reduces ferredoxin (Fd) and NAD<sup>+</sup>. The electron-bifurcating caffeyl-coenzyme A reductase (CarCDE) catalyzes the reduction of Fd and caffeyl-coenzyme A with NADH. As during growth on H<sub>2</sub> + CO<sub>2</sub> (Figure 2), energy is conserved by Rnf plus ATP synthase (both Na<sup>+</sup> dependent). Only reactions in the steady state of caffeate respiration are shown.

hydrolyzed by a membrane-bound pyrophosphatase, coupled to Na<sup>+</sup> translocation (7). In the steady state of caffeate respiration, a coenzyme A transferase takes over the job of caffeate activation by transferring the CoA from hydrocaffeyl-CoA to caffeate. Caffeyl-CoA is then reduced by the aforementioned electron-bifurcating caffeyl-CoA reductase, CarCDE. The Fd<sub>red</sub> then fuels the respiratory chain. NAD is reduced with H<sub>2</sub> by the electron-bifurcating hydrogenase, again leading to the reduction of Fd, which also goes into the respiratory chain. Caffeate respiration is a prime example for a metabolic scenario in which electron bifurcation is essential for energy conservation since it is the only mechanism by which the fuel for respiration is generated. Again, electron bifurcation per se is not energy conserving, but it generates the fuel for energy conservation.

A third example is lactate oxidation by *A. woodii* (89, 113) (Figure 4). Lactate is a common compound in anaerobic ecosystems produced by primary fermenters such as lactic acid bacteria. The lactate/pyruvate couple ( $E_0' = -190$  mV) has a very unfavorable redox potential for the reduction of NAD<sup>+</sup>. There are two solutions to the problem. Facultative anaerobes such as *E. coli* have a membrane-bound lactate dehydrogenase (Ldh) that transfers electrons to a ubiquinone ( $E_0' = -113$  mV). The enigma of how strict anaerobes without cytochromes oxidize lactate was solved recently (89, 113). The steep energy barrier is overcome by an electron-bifurcating Ldh/Etf complex that uses Fd<sub>red</sub> as coreductant according to



6.

**Figure 4**

Acetogenesis from lactate as carried out by *Acetobacterium woodii*. The endergonic oxidation of lactate is driven by concomitant oxidation of  $\text{Fd}^{2-}$  catalyzed by the electron-bifurcating Ldh-EtfAB complex.  $\text{Fd}^{2-}$  is provided by the Rnf complex under the exploitation of a  $\text{Na}^+$  gradient.  $\text{CO}_2$  is reduced in the Wood-Ljungdahl pathway. Energy is conserved by substrate level phosphorylation (through acetate kinase). Abbreviations: CODH/ACS, carbon monoxide dehydrogenase/acetyl-CoA synthase; CoFeSP, corrinoid-iron-sulfur protein; Fd, ferredoxin; HDCR, hydrogen-dependent carbon dioxide reductase; Ldh, lactate dehydrogenase; THF, tetrahydrofolate.

Pyruvate is decarboxylated to acetyl-CoA and  $\text{CO}_2$  under the reduction of Fd. The reducing equivalents generated are consumed by  $\text{CO}_2$  reduction. However, there is a need for reducing Fd with NADH as reductant. Fd is reduced with NADH by reverse electron transport, catalyzed by the Rnf complex. This reaction is driven by the electrochemical sodium ion potential across the membrane, established by ATP hydrolysis. In addition, FBEB is essential to generate  $\text{H}_2$  from NADH. This metabolic scenario is not only an example for electron bifurcation as a driving force for oxidizing an organic substrate, but also an example for reverse chemiosmosis: ATP hydrolysis generates an electrochemical sodium ion potential that then drives reverse electron transport to reduce Fd, which then activates substrate oxidation. This is a good example of how electron bifurcation allows growth on low-energy substrates.

Another low-energy substrate is ethanol, with a redox potential for the ethanol/aldehyde pair of  $E_0' = -190 \text{ mV}$ . At least in *A. woodii*, substrate oxidation is driven not by electron bifurcation but by a bifunctional enzyme, AdhE, that couples ethanol and acetaldehyde oxidation, with the latter pulling the former (5). However, other anaerobes may use electron bifurcation for ethanol oxidation. It is proposed that in *Desulfovibrio vulgaris* growing on ethanol/sulfate, Adh oxidizes ethanol, which produces NADH that is finally oxidized by FlxABCD. Electrons are finally transferred to



HdrABC, which bifurcates electrons to Fd and (as proposed) DsrC (a cysteine-containing small protein) (81).

## ELECTRON BIFURCATION ALLOWS FOR ETHANOL PRODUCTION FROM CARBON DIOXIDE

Ethanol formation from  $\text{H}_2 + \text{CO}_2$  according to



needs 0.1 ATP to proceed in *A. woodii* (2), and ethanol formation from  $\text{H}_2$  and  $\text{CO}_2$  was not reported for that species. A major problem is that acetyl-CoA is reduced to ethanol rather than metabolized to acetate, yielding less ATP. Interestingly, however, the mesophilic acetogenic bacterium *Clostridium autoethanogenum* forms ethanol and acetate in a ratio of ~1:1 during growth on  $\text{H}_2 + \text{CO}_2$ . Ethanol formation is possible by the involvement of two electron-bifurcating enzymes: transhydrogenase, which catalyzes NADPH oxidation coupled to the concomitant reduction of  $\text{NAD}^+$  and Fd by the reversal of Reaction 11 (see the section below titled Bifurcating Enzymes), and methylenetetrahydrofolate reductase. So far, the bifurcating activity of the latter enzyme has not been demonstrated; however, the enzyme does not carry out NADH- or NADPH-dependent reduction of methylenetetrahydrofolate and only uses viologen dyes as electron acceptors. Energetically, however, it is crucial in this scenario that this enzyme provide additional  $\text{Fd}_{\text{red}}$ , as this allows for additional energy conservation via the Rnf complex in the form of a proton gradient.

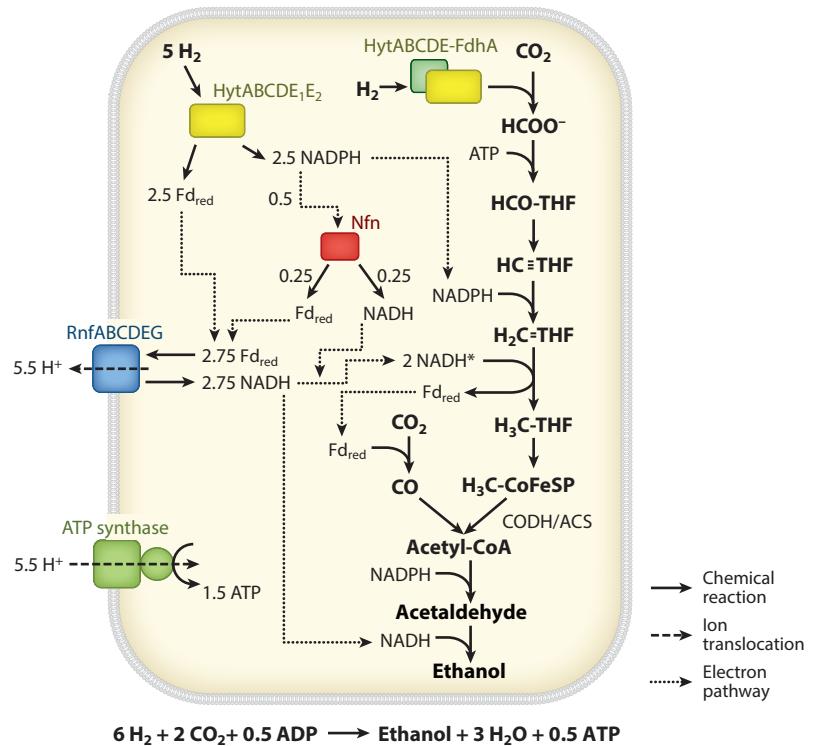
Measurement of key enzyme activities in the cell extracts of *C. autoethanogenum* revealed other important biochemical differences to *A. woodii* (71). One of the most important differences is that key enzymes are NADP specific, such as the bifurcating HytABCDE<sub>1</sub>E<sub>2</sub> complex (see section below titled Bifurcating Enzymes), which provides NADPH and  $\text{Fd}_{\text{red}}$  to the WLP for  $\text{CO}_2$  reduction but also for acetyl-CoA reduction. In consequence, in the WLP, NADP<sup>+</sup>-specific methylenetetrahydrofolate dehydrogenase is NADPH dependent, and interestingly, NADP<sup>+</sup>-specific aldehyde dehydrogenase activity was found in cell-free extract of *C. autoethanogenum*. Assuming that methylenetetrahydrofolate reductase is bifurcating, ATP yield from ethanol formation with  $\text{H}_2$  would be positive in *C. autoethanogenum* (Figure 5).

Meanwhile, it has been demonstrated by genetic methods that in addition to the biochemical differences mentioned above, another nonbifurcating enzyme is essential for ethanol formation from  $\text{H}_2 + \text{CO}_2$ : an aldehyde:ferredoxin oxidoreductase (AOR; 71, 61). The enzyme is responsible for the direct reduction of acetate to acetaldehyde, which allows for the conservation of more ATP per ethanol formed, as conversion of acetyl-CoA to acetate yields additional ATP (by the acetate kinase reaction).

## A COMPARISON OF THE THREE COUPLING MODES: ELECTRON BIFURCATION, REVERSE ELECTRON TRANSPORT, AND ATP HYDROLYSIS

Electron bifurcation is a direct coupling mechanism that is energetically superior to the two other modes. For the following comparison, we consider the endergonic reduction of Fd ( $E_0' = -500$  mV) with  $\text{H}_2$  ( $E_0' = -414$  mV) as reductant. The redox potential difference of 0.086 V corresponds to a free energy change of 8.3 kJ/mol that needs to be invested. If the reaction is coupled directly to ATP hydrolysis, 1 ATP equivalent to 60 kJ/mol has to be invested under cellular conditions ( $\Delta G_P' = -60$  kJ/mol). For an indirect coupling mechanism via reverse electron

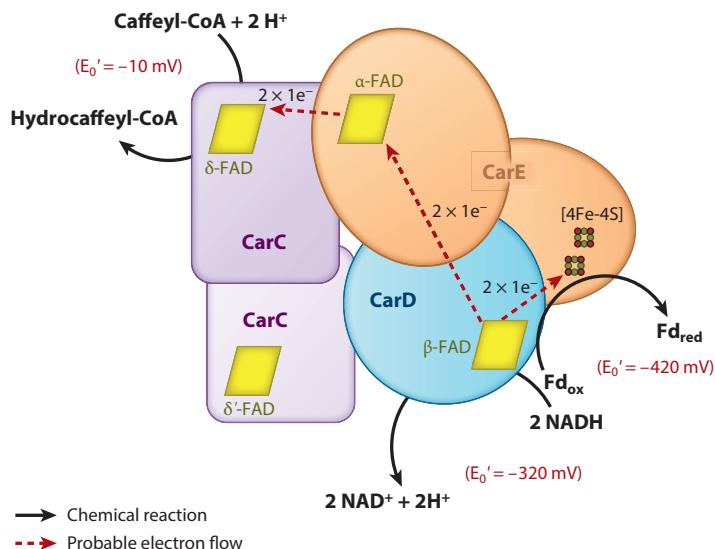


**Figure 5**

Ethanol formation from  $\text{H}_2 + \text{CO}_2$  as carried out by *Clostridium autoethanogenum*. Hydrogen is used for the reduction of  $\text{CO}_2$  in the WLP by the formate dehydrogenase FdhA in a complex with the bifurcating hydrogenase HytABCDE<sub>1</sub>E<sub>2</sub>. Hydrogen is also oxidized by HytABCDE<sub>1</sub>E<sub>2</sub>, providing  $\text{Fd}_{\text{red}}$  and NADPH. NADPH is used as reductant for the reduction of methenyltetrahydrofolate in the WLP and for acetyl-CoA reduction. NADH for the reduction of acetaldehyde and for methylenetetrahydrofolate is provided by the Rnf complex and by the transhydrogenase Nfn. For simplicity, the model does not account for aldehyde:ferredoxin oxidoreductase, which has been demonstrated to be essential in ethanol formation in *C. autoethanogenum* (61). Abbreviations: CoFeSP, corrinoid–iron–sulfur protein; Fd, ferredoxin;  $\text{Fd}_{\text{red}}$ , reduced Fd; THF, tetrahydrofolate; WLP, Wood-Ljungdahl pathway.

transport we consider as a first step an electron transfer from  $\text{H}_2$  to  $\text{NAD}^+$  ( $E_0' = -320 \text{ mV}$ ), as catalyzed by a typical NiFe hydrogenase. Next, NADH is oxidized by a membrane-bound enzyme complex, for example Rnf, and endergonic electron transfer to Fd is driven by  $\text{Na}^+/\text{H}^+$  influx. The energy required to drive reverse electron flow of two electrons over a difference of 180 mV to Fd is 35 kJ/mol, which is equivalent to 0.58 mol ATP. If the reaction is coupled to  $\text{H}_2$ -dependent  $\text{NAD}^+$  reduction by electron bifurcation, 80 mV potential difference is invested, equivalent to 0.23 ATP. Electron bifurcation requires the least energy of all three modes of coupling. At a first glance, the differences between the three mechanisms seem not very dramatic, but they are indeed physiologically relevant. For example, investment of 0.5 mol ATP for  $\text{H}_2$  activation in acetogens would make acetogenesis according to Equation 4 impossible! Clearly, energy coupling by electron bifurcation allows for growth on low-energy substrates and thus may be an ancient mechanism allowing lithotrophic growth of first organisms such as methanogens or acetogens on early Earth.



**Figure 6**

Schematic representation of the structure and function of the *Acetobacterium woodii* CarCDE complex. The interaction of NADH, ferredoxin (Fd) and CarC with CarDE is representative of the crystal structure of the CarCDE complex. CarE consists of domains I and II (orange); CarC is a homodimer (dark and light purple); CarD (blue) docks onto one subunit of CarC (light purple), while CarE donates electrons to the other subunit of CarC (dark purple). However, direct involvement of [4Fe-4S] of CarE in electron transfer to Fd has not been established yet.

## BIFURCATING ENZYMES

### Electron-Bifurcating Acyl-CoA Dehydrogenases

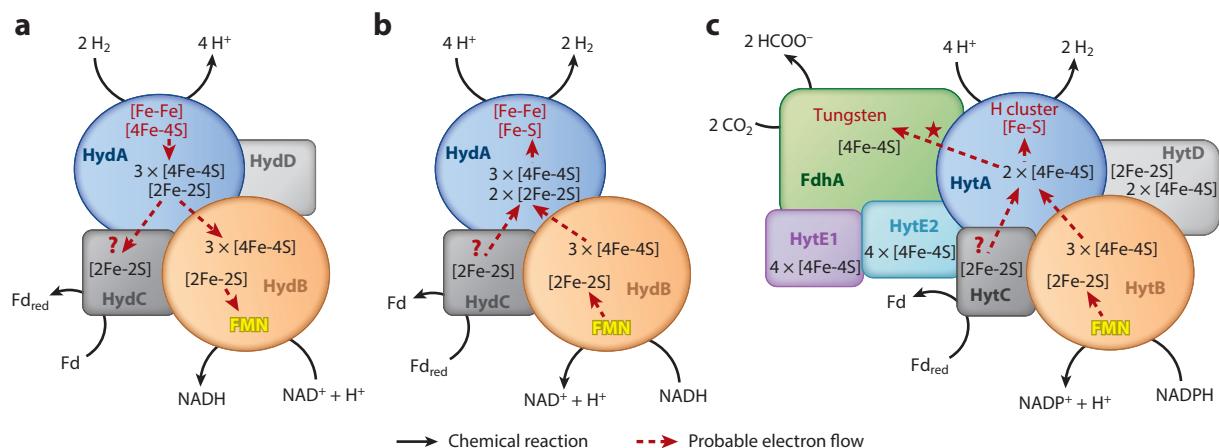
The electron bifurcating Bcd complex from *C. kluyveri* is apparently widespread in clostridia; for example, it is also found in the acetogenic bacterium *Eubacterium limosum* (55). A similar enzyme is present in the acetogenic bacterium *A. woodii*, where it is involved in caffate respiration (see above). The caffeyl-CoA reductase CarCDE is very similar to the Bcd/Etf complex of *C. kluyveri* and catalyzes the following reaction:



*carC* encodes the dehydrogenase, and *carD* and *carE* encode EtfA and EtfB (4). CarD harbors the NADH binding site, CarE the Fd, and CarC the caffeyl-CoA binding site. The structure was solved recently (20). In contrast to the Bcd/Etf complex, the CarCDE complex harbors additional FeS clusters in a Fd-like domain in CarE, and it was speculated that the CarCDE complex may directly interact with the Rnf complex via this domain (Figure 6).

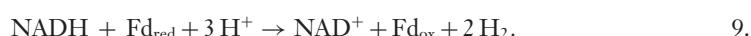
### Electron-Bifurcating Hydrogenases

The redox potential of the NADH/NAD couple is too electropositive ( $E_{0'} = -320$  mV) to reduce protons ( $E_{0'} = -414$  mV). In 2009, Schut & Adams (95) isolated a FeFe-hydrogenase from *Thermotoga maritima* that uses FBEB to drive the endergonic formation of H<sub>2</sub> directly from

**Figure 7**

Schematic representation of the structure and function of three electron-bifurcating hydrogenases. (a) HydABCD from *Acetobacterium woodii* is involved in hydrogen uptake. (b) HydABC hydrogenase from *Thermotoga maritima* catalyzes H<sub>2</sub> formation from NADH and Fd<sub>red</sub>, operating as an electron-confurcating hydrogenase. (c) HytABCDE<sub>1</sub>E<sub>2</sub> from *Clostridium autoethanogenum*, which is involved in CO<sub>2</sub> reduction to formate. Note that the latter complex catalyzes either the reduction of H<sup>+</sup> to H<sub>2</sub> or the reduction of CO<sub>2</sub> to formate. Question marks indicate that there is no experimental evidence, and the red star signifies the second route of electron flow to formate dehydrogenase. Abbreviations: Fd, ferredoxin; Fd<sub>red</sub>, reduced Fd; FMN, flavin mononucleotide.

NADH using Fd<sub>red</sub> as coreductant in one soluble enzyme:



The enzyme has three subunits, HydABC, and in vivo couples H<sub>2</sub> production from NADH and Fd<sub>red</sub> by a reversal of electron bifurcation, or electron confurcation (Figure 7). HydA harbors the H cluster that catalyzes H<sub>2</sub> oxidation and five FeS clusters, HydB carries a flavin mononucleotide and four FeS clusters, and HydC is a small protein carrying one 2Fe-2S center (95). The enzyme is, thus, more complex than the single subunit, Fd-dependent hydrogenase usually found in clostridia such as *C. pasteurianum*. An enzyme catalyzing the same reaction with an even more complex subunit composition was purified from *A. woodii* (92). It contained four subunits, HydABCD; HydABC are similar in cofactor content to the HydABC from *T. maritima* (95, 106). The additional subunit HydD has no predicted cofactors and thus is unlikely to be involved in electron transport. The *hyd* gene cluster in *A. woodii* encodes an additional protein, HydE, which is apparently not part of the complex and may be involved in biogenesis of the enzyme.

In *A. woodii*, the enzyme is involved in H<sub>2</sub> uptake as well as in H<sub>2</sub> evolution, and thus during autotrophic and during heterotrophic growth. A variation of these hydrogenases is present in *C. autoethanogenum* (71, 94, 109). There, the hydrogenase does not reduce NAD but NADP (and Fd) and is composed of six subunits HytABCDE<sub>1</sub>E<sub>2</sub>. Apparently, it is involved in CO<sub>2</sub> reduction to formate with H<sub>2</sub> as reductant (110) according to the following:



Electron-bifurcating hydrogenases operating in the confurcation mode are a prime example of FBEB used as a third type of energy coupling. It is well known that aerobic chemolithotrophs growing on substrates with redox potentials much more positive than NAD/NADH, for example Fe<sup>2+</sup>, use reverse, membrane-bound electron transport driven by the electrochemical ion gradient



across the membrane to reduce NAD (28). In the case of electron-confurcating hydrogenases, it is obvious that  $\Delta\tilde{\mu}_{H^+}$  has been replaced by electron bifurcation as driving force.

### Electron-Bifurcating Transhydrogenases

Transhydrogenases are essential to every living cell to balance the cellular redox pools of NAD and NADP<sup>+</sup> (56, 79, 80). In *C. kluyveri*, the enzyme is needed at low concentrations of the substrates ethanol and acetate, while in *C. autoethanogenum* it is involved in redox metabolism during ethanol formation from H<sub>2</sub> + CO<sub>2</sub> (**Figure 5**). The midpoint potential of NADH under cellular conditions is around -300 mV and that of NADPH is -360 mV (109). Thus, NADH-dependent reduction of NADP<sup>+</sup> requires energy, provided for example by a transmembrane electrochemical ion gradient across the cytoplasmic membrane in the case of the membrane-bound transhydrogenase. The Nfn complex of *C. kluyveri* (112) is a soluble transhydrogenase that instead uses electron bifurcation to couple the endergonic reaction to an exergonic reaction. Fd<sub>red</sub> is the driving force according to



Again, electron bifurcation replaces  $\Delta\tilde{\mu}_{H^+}$  as driving force.

NfnAB of *C. kluyveri* is a heterodimer consisting of subunit A (~32 kDa) and subunit B (~50 kDa). NfnA contains 1 FAD and 1 [2Fe-2S] cluster and NfnB 1 FAD (bifurcating site) and 2 [4Fe-4S] clusters. NADPH is the electron donor that binds to NfnB; 1 electron is transferred to the [2Fe-2S] in Nfn A and then to FAD, which reduces NAD<sup>+</sup>. The other electron flows via the 2 [4Fe-4S] clusters and reduces the Fd. This process is repeated twice, utilizing 2 NADPH to reduce 1 NAD<sup>+</sup> and 1 Fd (21, 112). The reaction is completely reversible according to Reaction 11.

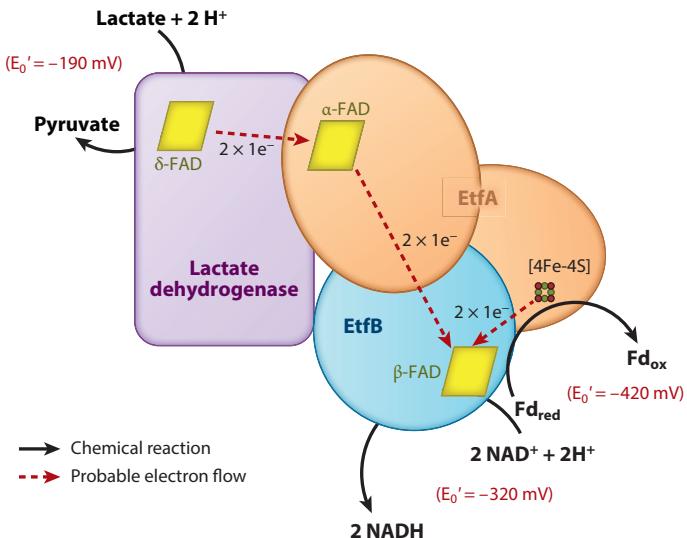
### Electron-Bifurcating Lactate Dehydrogenases

The lactate dehydrogenase from the acetogenic model bacterium *A. woodii* has been purified (113). The Ldh formed a stable complex with an electron-transferring flavoprotein (Etf) and catalyzed lactate oxidation according to Reaction 6. The enzyme did not oxidize lactate with NAD, unless Fd<sub>red</sub> was added. Fd oxidation and NAD reduction were linked stoichiometrically 1:1. The Ldh/Etf complex harbors 3 predicted FADs and 1 [4Fe-4S] cluster bound (**Figure 8**). FAD was required for enzyme stability and activity, and FMN could not substitute for FAD. The enzyme complex also catalyzed the reverse reaction, the thermodynamically favorable pyruvate reduction to lactate with NADH as electron donor, but only if Fd<sub>red</sub> was present. *EtfAB* genes in genomic proximity to *ldh* can be found in many anaerobes, indicating wide use of FBEB to drive lactate oxidation.

### Electron-Bifurcating Heterodisulfide Reductase

Methanogenic archaea reduce CO<sub>2</sub> to methane with H<sub>2</sub>, formate, CO, or methyl groups as reductant (101, 102). In addition, some can disproportionate acetate (30). Common to all substrates is the liberation of methane from methyl-CoM by a nucleophilic attack of the thiolate anion of coenzyme B (CoB) (7-mercaptopheptanoylthreonine phosphate), giving rise to methane and a mixed disulfide of CoM and CoB. In the last step of methanogenesis, this disulfide needs to get reduced to the corresponding thiols (29). Methyl-CoM reductase catalyzes the reaction between methyl-CoM and CoB and releases methane and heterodisulfide, which is further reduced to HS-CoM and HS-CoB by heterodisulfide reductase (HdrABC) (39, 40, 96). In methanogens without cytochromes this is catalyzed by an electron-bifurcating heterodisulfide reductase (57,



**Figure 8**

Schematic representation of the structure and function of the *Acetobacterium woodii* Ldh-Etf complex. The interaction of NADH, Fd, and Ldh with Etfo is assumed by comparison to the crystal structure of the CarCDE complex. Therefore, pyruvate reduction to lactate is shown; however, the enzyme is fully reversible. Abbreviations: Etfo, electron-transferring flavoprotein; Fd, ferredoxin; Etfo<sub>ox</sub>, oxidized ferredoxin; Ldh, lactate dehydrogenase.

117). This cytoplasmic enzyme couples (a) exergonic electron flow from H<sub>2</sub> to the heterodisulfide to (b) endergonic electron flow from H<sub>2</sub> to Fd. The recently published crystal structure of the HdrABC- [Ni-Fe]-hydrogenase from *M. thermolithotrophicus* (108) reveals HdrB contains 2 noncubane [4Fe-4S] clusters composed of fused [3Fe-4S]-[2Fe-2S] sharing 1 iron and 1 sulfur. HdrABC in total contains 8 [4Fe-4S] and 1 FAD. Recently, a novel Hdr from *Methanosarcina acetivorans* was described that uses reduced F<sub>420</sub> as electron donor for the endergonic reduction of Fd, coupled to the exergonic reduction of the heterodisulfide (117).

## THE MOLECULAR MECHANISM OF FBEB

Until now, the structures of four electron-bifurcating proteins had been solved to atomic resolution: the Nfn transhydrogenase from *C. kluyveri* (21) and *P. furiosus* (63), the Bcd/Etf complex from *Acidaminococcus fermentans* (18) and *Clostridium difficile* (22), the CarCDE complex from *A. woodii* (20), and the heterodisulfide reductase from *M. thermolithotrophicus* (107). In all bifurcating enzyme complexes, FAD plays the central role in shifting the electrons toward endergonic and exergonic pathways. The literature is exploding with publications on the molecular basis of electron bifurcation, based on not only the structures but also kinetic and biophysical measurements (4, 16–19, 21–23, 46, 47, 57, 58, 60, 63, 71, 83, 92, 108–110, 112, 113). Here, we discuss briefly the molecular mechanism of FBEB with respect to the butyryl-CoA dehydrogenase complex found in clostridia. The Etfo/Bcd complex is very similar to the electron-bifurcating caffeyl-CoA reductase (4), to the lactate dehydrogenase (113) from acetogens and to FixAB (58) from nitrogen-fixing *Azotobacter vinelandii*. Interestingly, in acetogens, an extra Fd-like domain is fused to the N terminus of EtfoA. Deletion of this domain, however, does not result in the loss of bifurcation activity (20).



Electron-bifurcating Etf/Bcd is a heterotrimeric protein complex. Etf is formed by  $\alpha$  and  $\beta$  subunits, whereas the  $\delta$  subunit is the Bcd. Each subunit contains 1 FAD cofactor known as  $\alpha$ -FAD,  $\beta$ -FAD, and  $\delta$ -FAD, respectively. EtfAB consists of three domains, I and II being part of the Etf $\alpha$  subunit and domain III forms the Etf $\beta$ . The highly mobile domain II holds  $\alpha$ -FAD, and  $\beta$ -FAD is located in a cleft made by domains I and III. The 2 FADs are separated by a distance of 18 Å; interestingly, a rearrangement of domain II toward domains I and III reduces the distance to 14 Å without disturbing protein architecture (18). NAD(P)H binds next to the Etf $\beta$ -FAD and reduces it by a hydride transfer ( $\beta$ -FADH $^-$ ). Subsequently, electrons are transferred sequentially to corresponding dehydrogenase ( $\delta$ -FAD) and Fd.

The recently resolved crystal structure of the Etf/Bcd complex from *C. difficile* (22) reveals a different orientation of domain II where  $\alpha$ -FAD is close to  $\delta$ -FAD of a Bcd subunit (8.5 Å), called the dehydrogenase contact state (D-state). In the D-state, the distance between  $\alpha$ - and  $\beta$ -FAD remains 37 Å, rendering a direct electron transfer within the 2 FADs impossible. Superimposition of the two crystal structures of Etf from *A. fermentans* and *C. difficile* allows a model building for FBEB. When two flavins are 14 Å apart after domain reorientation, the state is called bifurcation-like state (B-state). Electron bifurcation by FAD is mediated by stepwise electron transfer into two different redox potential levels. One necessity for the bifurcating FAD is that it should exhibit a crossed redox potential, i.e., the potential of the flavin semiquinone/hydroquinone (SQ/HQ) couple should be more positive than that of quinone/semiquinone (Q/SQ) (1, 78). The crossed potential ensures electron transfer from HQ toward the high potential electron acceptor (crotonyl-CoA,  $E_0' = -10$ mV), which allows the remaining SQ to attain a very low redox potential that reduces low potential acceptor (Fd,  $E_0' = -420$ mV) (Figure 9).

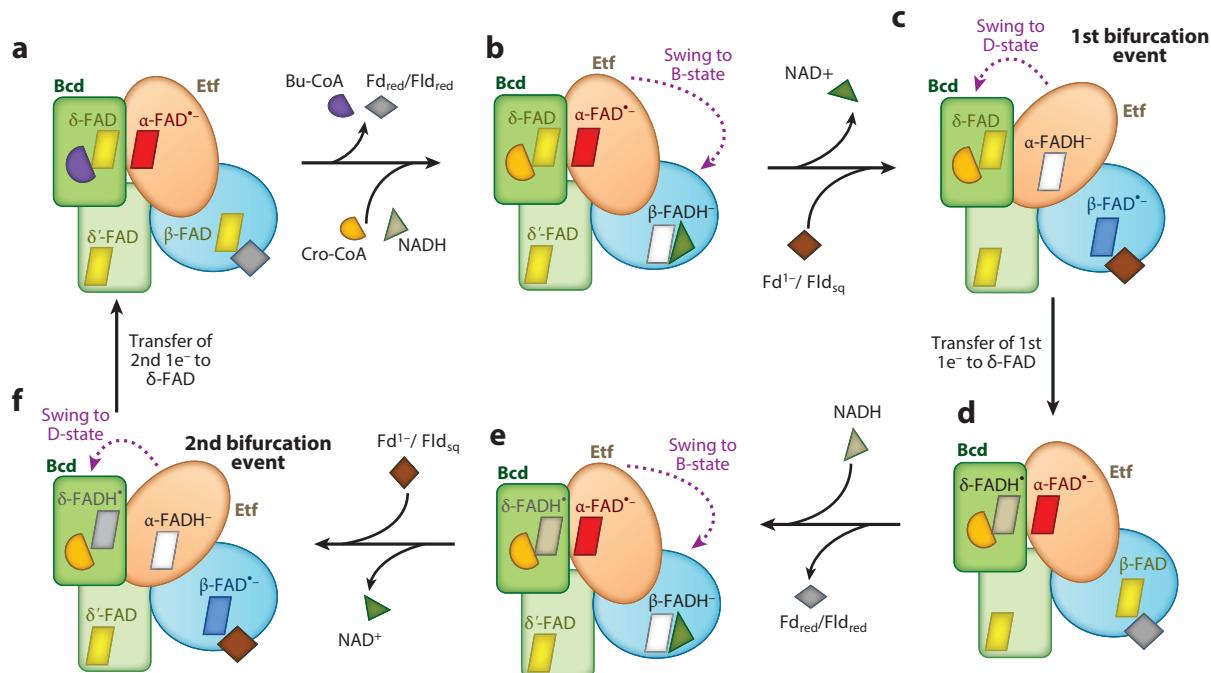
FBEB starts with the fully reduced  $\beta$ -FADH $^-$  (on reduction of  $\beta$ -FAD by hydride transfer from NADH). Domain II swings over to the B-state, allowing a single electron to be transferred to  $\alpha$ -FAD $^{\circ-}$  (considered to be half reduced in vivo under steady state) (21). As soon as the first electron leaves the  $\beta$ -FADH $^-$ , a low-potential  $\beta$ -FADH $^\circ$  or  $\beta$ -FAD $^{\circ-}$  ( $E_0' = -700$ mV) is formed that quickly reduces Fd $^{1-}$  or Fld $^{1-}$  (flavodoxin) ( $E_0' = -420$ mV). Domain II then flips over to the D-state and transfers one electron to the  $\delta$ -FAD of the Bcd. In this first complete round of bifurcation, 1 NADH is oxidized to semireduce the  $\delta$ -FAD to  $\delta$ -FADH $^\circ$  and completely reduce Fd. In the second round of bifurcation, the binding of another molecule of NADH triggers  $\alpha$ -FAD $^-$  to flip over once again to the B-state. It collects 1 electron and swings back to generate  $\delta$ -FADH $^-$ , which reduces the crotonyl-CoA to butyryl-CoA. At the end of a complete bifurcation process, 2 molecules of NADH are oxidized to reduce 1 molecule of crotonyl-CoA and 2 Fd $^{1-}$  or Fld $_{\text{sq}}$ . The sequence of events during a bifurcation process is shown in Figure 10.

One interesting aspect was found in the bifurcating Etf/Bcd complex from *Megasphaera elsdenii* (16). Under aerobic conditions, Fd (69) was replaced by molecular oxygen, which was finally reduced to toxic oxygen radicals and hydrogen peroxide with NADH. However, on addition of the product (butyryl-CoA), NADH was completely oxidized and a higher concentration of hydrogen peroxide was formed. This showed that in the presence of oxygen, bifurcation may lead to the formation of reactive oxygen species, which may in turn trigger the oxidative stress response in anaerobic bacteria (52, 65, 68, 87, 88, 98, 116).

## FERREDOXIN, AN ANCIENT ENERGY CURRENCY?

As outlined above, catabolism is connected to anabolism by ATP that is generated by the two mechanisms of substrate-level phosphorylation and chemiosmosis. Ancient methanogens without cytochromes synthesized ATP by a chemiosmotic mechanism with methyltetrahydromethanopterin:CoM methyltransferase as a sodium ion pump (33, 72, 76) and Na $^+$ -A<sub>1</sub>A<sub>O</sub>-ATP



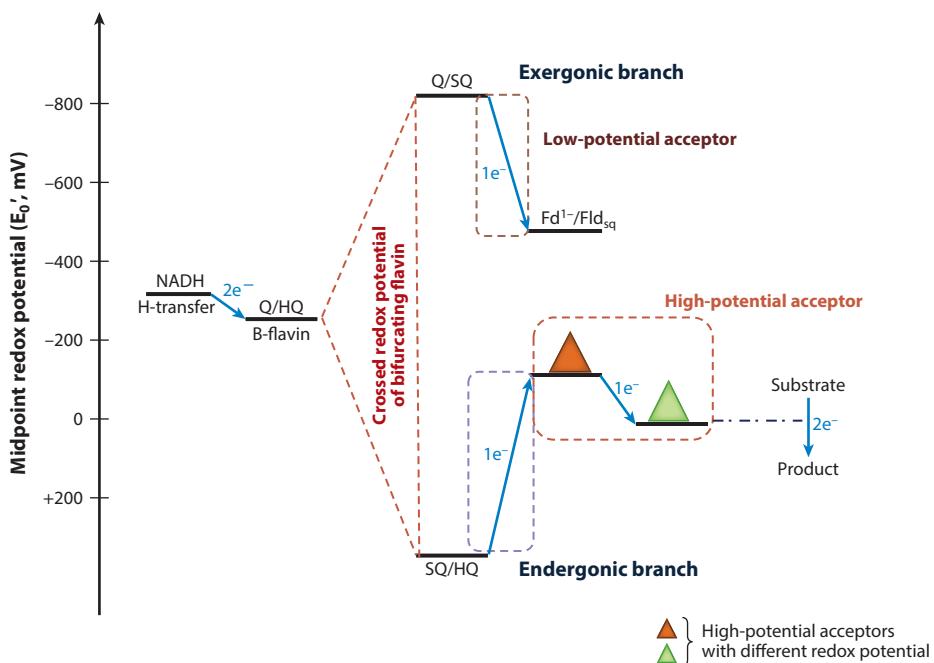
**Figure 9**

Ballet of flavins. Mechanism of the flavin-based electron-bifurcation process of the Etf/Bcd complex on the basis of the crystal structure from *Clostridium difficile*, modified from Reference 22. The figure shows the Bcd dimers (green and light green) interacting with Etf (light blue, domain I/III; beige, domain II). The small trapezoids depict FAD in the quinone state (yellow), anionic semiquinone state (red), neutral semiquinone state (light blue in Etf and gray in Bcd), and hydroquinone state (white). The cycle starts at panel *a*; with all FADs oxidized, butyryl-CoA and Fd<sub>red</sub> are exchanged by incoming NADH and crotonyl-CoA. (*b*) Bound NADH reduces β-FAD to hydroquinone β-FADH<sup>-</sup>, and domain II swings over toward β-FAD (B-state) located at domain I/III. α-FAD° picks up 1 electron, leaving behind β-FAD<sup>°-</sup>. (*c*) NAD<sup>+</sup> is released and is replaced by the Fd, and domain II swings over to the D-state and transfers 1 electron to δ-FAD. (*d*) Fd is reduced by a single electron, 1 electron is transferred from α-FADH<sup>-</sup> to δ-FAD, and Fd is released by incoming NADH. The sequence of reactions from panel *a* to panel *d* completes the first round of bifurcation. The same sequence is repeated in panels *e-a* for the second round of bifurcation. In the second round δ-FAD° is fully reduced by 1 incoming electron from α-FADH<sup>-</sup> that is further transferred to the substrate (crotonyl-CoA) to reduce it to butyryl-CoA (product). The red semiquinone of the α-FAD at the onset of bifurcation *in vivo* has been predicted but not tested. Appearance of a stabilized red semiquinone of α-FAD° has been shown earlier with a stoichiometric concentration of NADH. Abbreviations: Bu, butyryl; Cro, crotonyl; Fd, ferredoxin; Fd<sub>red</sub>, reduced Fd; Fld, flavodoxin; Fld<sub>sq</sub>, Fld in semiquinone state.

synthase (35, 66, 74). In addition, the catabolic heterodisulfide reductase is coupled to reduction of Fd by FBEB, and Fd<sub>red</sub> is then the driving force for the first step in CO<sub>2</sub> fixation. In addition, Fd<sub>red</sub> is the driving force for carboxylation of acetyl-CoA to pyruvate, the key reaction in biosynthesis of organic matter from CO<sub>2</sub> via acetyl-CoA. Although ATP is still the major energy currency, at least to some extent, Fd can substitute for ATP. This may open the possibility that Fd has been an ancient energy currency in the absence of ATP. If this is true, then electron bifurcation may indeed have been a mechanism of energy conservation in ancient organisms yet to be discovered.

However, it has to be considered that ATP and  $\Delta\bar{\mu}_{H^+}/\Delta\bar{\mu}_{Na^+}$  are ideal cellular energy currencies that drive all three types of work of a cell: chemical, osmotic (substrate transport), and physical (motility). It is hardly imaginable how Fd<sub>red</sub> should drive, for example, the latter two. But motility is dispensable and transport processes may be less important in a primordial world. Fd (FeS clusters) may be a relic of an ancient, simple metabolism.



**Figure 10**

Schematic representation of the proposed electron flow during bifurcation via crossed redox potential of the bifurcating FAD. The quinone form of the bifurcating FAD (B-flavin) is reduced with two electrons from NADH to yield the hydroquinone form. The redox potential of the hydroquinone/semiquinone pair only allows for the reduction of the high-potential acceptor and constitutes an uphill electron flow. Subsequently, the remaining electron reduces the low-potential acceptor Fd, representing a downhill electron flow and rendering the complete reaction exergonic. Abbreviations: Fd, ferredoxin; Fld, flavodoxin; Fld<sub>sq</sub>, flavodoxin in semiquinone state; HQ, hydroquinone; Q, quinone; SQ, semiquinone.

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