Review

Energy conservation via electron bifurcating ferredoxin reduction and proton/Na⁺ translocating ferredoxin oxidation

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A B S T R A C T

The review describes four flavin-containing cytoplasmatic multienzyme complexes from anaerobic bacteria and archaea that catalyze the reduction of the low potential ferredoxin by electron donors with higher potentials, such as NAD(P)H or H₂. These endergonic reactions are driven by concomitant oxidation of the same donor with higher potential acceptors such as crotonyl-CoA, NAD⁺ or heterodisulfide (CoM-S-S-CoB). The process called flavin-based electron bifurcation (FEBB) can be regarded as a third mode of energy conservation in addition to substrate level phosphorylation (SLP) and electron transport phosphorylation (ETP). FBE has been detected in the clostridial butyryl-CoA dehydrogenase/electron transferring flavoprotein complex (BcdA-EtfBC), the multisubunit [FeFe]hydrogenase from Thermotoga maritima (HydABC) and from acetogenic bacteria, the [NiFe]hydrogenase/heterodisulfide reductase (MvhADG–HdrABC) from methanogenic archaea, and the transhydrogenase (NfnAB) from many Gram positive and Gram negative bacteria and from anaerobic archaea. The BcdA/Etf complex that catalyzes electron bifurcation from NADH to the low potential ferredoxin and to the high potential crotonyl-CoA has already been studied in some detail. The bifurcating protein most likely is EtfBC, in which each subunit (βγ) contains one FAD. In analogy to the bifurcating complex III of the mitochondrial respiratory chain and with the help of the structure of the human ETF, we propose a conformational change by which γ-FADH⁻ in EtfBC approaches β-FAD to enable the bifurcating one-electron transfer. The ferredoxin reduced in one of the four electron bifurcating reactions can regenerate H₂ or NADPH, reduce CO₂ in acetogenic bacteria and methanogenic archaea, or is converted to ΔμH⁺/Na⁺ by the membrane-associated enzyme complexes Rnf and Ech, whereby NADH and H₂ are recycled, respectively. The main bacterial Rnf complexes couple ferredoxin oxidation by NAD⁺ with proton/sodium ion translocation and the more diverse energy converting [NiFe]hydrogenases (Ech) do the same, whereby NAD⁺ is replaced by H⁺. Many organisms also use Rnf and Ech in the reverse direction to reduce ferredoxin driven by ΔμH⁺/Na⁺. Finally, examples are shown, in which the four bifurcating multienzyme complexes alone or together with Rnf and Ech are integrated into energy metabolisms of nine anaerobes. This article is part of a Special Issue entitled: The evolutionary aspects of bioenergetic systems.

1. Introduction

The electron carrier ferredoxin is involved in the energy metabolism of many anaerobes e.g. clostridia, acetogenic and sulfate reducing bacteria as well as methanogenic archaea. Ferredoxins are cytoplasmatic, acidic iron–sulfur proteins with either one [2Fe–2S] cluster or one, two or more [4Fe–4S] clusters each of which can be reduced by one electron. The redox potentials E°⁺ of ferredoxins are close to that of the hydrogen (H₂) electrode at pH 7.0 which is −414 mV. This reflects that in most energy metabolisms, in which ferredoxins are involved, dihydrogen is either a substrate or a product. Within the cells the ferredoxins are more than 90% reduced allowing them to serve as electron donors in reactions with standard redox potentials (E°⁻) as low as or even below −500 mV. Under conditions of iron deprivation many anaerobes synthesize flavodoxins instead of ferredoxins and the flavodoxins substitute for the ferredoxins in most functions (Table 1) [1–5].

Until recently the following catalytic ferredoxin-dependent redox reactions were known, some of which can function in vivo in both directions (not complete): the reduction of protons to H₂ (E°⁻ = −414 mV) [14], the reversible oxidation of pyruvate to acetyl-CoA and CO₂ (E°⁺ = −500 mV) [15], the reversible oxidation of CO to CO₂ (E°⁺ = −520 mV) [16], the reversible oxidation of formyl-methanofuran to...
CO₂ and methanofuran (E° = −500 mV) [12], the oxidation of acetaldheyde to acetic acid (E° = −580 mV) [17], the reversible oxidation of formate to CO₂ (E° = −430 mV) [18] and the oxidation of 3-phosphoglyceraldehyde to 3-phosphoglycerate (E° = −580 mV) [19]. These reactions are catalyzed by cytoplasmic iron–sulfur proteins some of which additionally contain thiamine pyrophosphate (pyruvate: ferredoxin oxidoreductase), nickel (CO dehydrogenase), molybdenum coordinating molybdenum and tungsten (formylmethanofuran dehydrogenase, acetaldheyde: ferredoxin oxidoreductase, formate: ferredoxin oxidoreductase and 3-phosphoglyceraldehyde: ferredoxin oxidoreductase). In addition reduced ferredoxin acts as electron donor for nitrogen fixation [20], activation of (R)-2-hydroxyacyl-CoA dehydratases [21], and reduction of benzoyl-CoA by the ATP-dependent [22] and independent enzyme systems (see Section 2.5).

In an energy metabolism for every ferredoxin-reducing reaction there must be at least one ferredoxin oxidizing reaction and these reactions must be stoichiometrically balanced. However, this was not always found to be the case, indicating that our knowledge with respect to ferredoxin-dependent reactions was not complete. Thus in the energy metabolism of Clostrium kluveri growing on ethanol and acetate, H₂ is formed in a ferredoxin-dependent reaction but there was no reaction known that can regenerate the reduced ferredoxin in vivo [11]. It was also not known, whether the free energy change associated with the re-oxidation of reduced ferredoxin with NAD⁺ or protons can be conserved. Both questions have in the meantime been resolved.

In the last few years two novel types of ferredoxin-dependent catalytic enzymes were found: (i) the cytoplasmic enzyme complexes coupling the endergonic reduction of ferredoxin with NADH, NADPH or H₂ to exergonic redox reactions via flavin-based electron bifurcation; and (ii) the membrane associated complexes RnfA-flavins to proteins strongly in

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### Table 1

<table>
<thead>
<tr>
<th>Oxidized cofactor/substrate</th>
<th>Reaction</th>
<th>E° (standard conditions)</th>
<th>E° (physiological conditions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferredoxin (Fd, clostridial type) [4,5]</td>
<td>Fd⁺ + e⁻ → Fd⁻</td>
<td>ca. −400 mV</td>
<td>ca. −500 mV</td>
</tr>
<tr>
<td>NAD⁺ [6]</td>
<td>NAD⁺ + 2 e⁻ + H⁺ → NADH</td>
<td>−320 mV</td>
<td>−280 mV</td>
</tr>
<tr>
<td>NADPH [6]</td>
<td>NAD⁺ + 2 e⁻ + H⁺ → NADH</td>
<td>−320 mV</td>
<td>−380 mV</td>
</tr>
<tr>
<td>FAD (or FMN) [7]</td>
<td>FAD⁺ + e⁻ + (H⁺) → FADH⁻</td>
<td>−172 mV</td>
<td>Variable</td>
</tr>
<tr>
<td>Flavodoxin (Fld) [8]</td>
<td>Fld⁺ + e⁻ + H⁺ → FldH⁻</td>
<td>−238 mV</td>
<td>Variable</td>
</tr>
<tr>
<td>ETF (porcine) [9]</td>
<td>ETF⁺ + e⁻ → ETF⁻</td>
<td>−205 mV</td>
<td>Variable</td>
</tr>
<tr>
<td>Crotonyl-CoA [10]</td>
<td>Crotonyl-CoA + 2 H⁺ + 2 e⁻ → butyryl-CoA</td>
<td>−10 mV</td>
<td>≤ −296 mV (≥ 10 Pa H₂)</td>
</tr>
<tr>
<td>CO₂ [11]</td>
<td>CO₂ + 2 e⁻ + H⁺ → CO + H₂O</td>
<td>−520 mV</td>
<td>−500 mV</td>
</tr>
<tr>
<td>CO₂/CO [11]</td>
<td>CO₂ + 2 e⁻ + H⁺ → CO + H₂O</td>
<td>−520 mV</td>
<td>−500 mV</td>
</tr>
<tr>
<td>CO₂/methanofuran (MFH) [12]</td>
<td>CO₂ + MFH + 2 H⁺ + 2 e⁻ → Formyl-MF + H₂O</td>
<td>−420 mV</td>
<td>−500 mV</td>
</tr>
<tr>
<td>CO₂/pyruvate [11]</td>
<td>CO₂ + Acetyl-CoA + H⁺ + 2 e⁻ → Pyruvate⁻ + CoA-SH</td>
<td>−500 mV</td>
<td>−500 mV</td>
</tr>
</tbody>
</table>

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a. The redox potentials of ferredoxins from different clostridia slightly vary.

b. Binding of flavins to proteins strongly influences their E°, see e.g. Fld and ETF.

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2. Cytoplasmic enzyme complexes coupling endergonic ferredoxin reduction reactions to exergonic redox reactions presumably via flavin-based electron bifurcation

In 2008 it was shown for the first time that in C. kluyveri the endergonic reduction of ferredoxin with NADH is coupled to the exergonic reduction of crotonyl-CoA to butyryl-CoA (Reaction (1)) and that this coupled reaction is catalyzed by a cytoplasmic enzyme complex [25]. Since then three more such coupled reactions catalyzed by cytoplasmic protein complexes were discovered (Reactions (2)–(4)) [26–28]. And this appears to be just the tip of an iceberg. Candidates for coupled reactions are the reduction of methyltetrahydrofolate to methylenetetrahydrofolate in acetogenic bacteria, the reduction of benzol-CoA in energy-limited anaerobic bacteria, the oxidation of formate in Clostridium acidici-urici, and the reduction of adenosine phosphosulfate (APS) as will be outlined in Section 2.5.

Fdo⁺ + 2NADH + crotonyl-CoA → Fdo⁻ + 2NAD⁺ + butyryl-CoA (1) \[ ΔG° = −44\text{k}_\text{j} /\text{mol} \]

Fdo⁺ + 2H₂ + NAD⁺ → Fdo⁻ + NADH + 3H⁺ (2) \[ ΔG° = −21\text{k}_\text{j} /\text{mol} \]

Fdo⁺ + 2H₂ + CoM-S–S–CoB → Fdo⁻ + CoM–SH + CoB–SH + 2H⁺ (3) \[ ΔG° = −50\text{k}_\text{j} /\text{mol} \]
FD₃₉₀ + 2NADPH + NAD⁺ ↔ FD⁺ + NADH + 2NADP⁺ + H⁺  \( \Delta G^o = +16kJ/mol. \)

Fd in Reactions (1)–(4) is the abbreviation for ferredoxin. Fd from \( C. pasteurianum \), which was used in most of the experiments, has a molecular mass of 6 kDa and a pi of 3.7 [1]. It contains two \([4Fe-4S]\) clusters both with a midpoint redox potential of near \(-400 mV\) [4] and this is the redox potential \( E^o \) used in the calculations of the free energy changes (\( \Delta G^o \)) associated with Reactions (1) to (4). The ferredoxins from \( A. fermentans \) and \( C. tetanomorphum \) have similar properties. Also used were \( E^o = -320 mV \) for the NAD⁺/NADH couple, \( E^o = -320 mV \) for the NADP⁺/NADPH couple, \( E^o = -10 mV \) for the crotonyl-CoA/butyryl-CoA couple [10] and \( E^o = -140 mV \) for the CoM-S-CoA/CoM-SH + CoB-SH couple (Table 1) [29].

For the understanding of the thermodynamics of Reactions (1) to (4) it is important to know that in living cells ferredoxins are generally more than 90% reduced (\( E = -500 mV \)), that NAD is more than 90% oxidized (\( E = -280 mV \)) and that the NAD⁺/NADPH ratio is 1/40 (\( E = -360 mV \)) [6]. Thus transhydrogenation from NADH (\( E = -280 mV \)) to NADP⁺ (\( E = -360 mV \)) in living cells requires energy [30] as does the reduction of ferredoxin (\( E = -500 mV \)) with H₂ at 10 Pa (\( E = -300 mV \)), which is the H₂ partial pressure in methanogenic habitats [29]. The redox potential \( E' \) of the \( \text{Fd}_{\text{red}}/\text{Fd}_{\text{ox}} \) couple was set at \(-500 mV\) because this is the redox potential at which in anaerobes many ferredoxin-dependent reactions operate. Examples are the pyruvate:ferredoxin oxidoreductase reaction (\( E^o = -500 mV \)) [11], the CO dehydrogenase reaction (\( E^o = -520 mV \)) [11] and the formylmethanofuran dehydrogenase reaction (\( E^o = -500 mV \)) [29].

The cytoplasmic enzyme complexes catalyzing Reactions (1)–(4) contain FAD or FMN. The butyryl-CoA dehydrogenase/electron transferring flavoprotein complex (BcdA–EtfBC complex) catalyzing Reaction (1) contains four FAD and no other prosthetic group. This is at present the strongest argument why we think that coupling of the endergonic reduction of ferredoxin with NADH, NADPH or H₂ is via flavin-based electron bifurcation in analogy to ubiquinone-based electron bifurcation discussed in Section 2.6. The \([FeFe]\) hydrogenase complex HydABC (Reaction (2)), the \([NiFe]\) hydrogenase–heterodisulfide reductase complex MvhADG/HdrABC (Reaction (3)) and the transhydrogenase complex NfnAB (Reaction (4)) contain besides FAD (HdrA and NfnAB) or FMN (HydAB) also many iron–sulfur clusters. They most likely have a function in electron transport but could equally well store electrons. Also, an iron sulfur cluster-based electron bifurcation mechanism, although not very likely, has to be considered. HydABC and NfnAB catalyze reversible reactions. They are electron confurcating rather than electron bifurcating when catalyzing the oxidation of reduced ferredoxin.

### 2.1. BcdA–EtfBC complex coupling ferredoxin reduction with NADH to crotonyl-CoA reduction

In 1971 it was reported that cell extracts from \( C. kluyveri \) catalyzed the reduction of ferredoxin with NADH only in the presence of acetyl-CoA and that concomitant to ferredoxin also acetyl-CoA was reduced [31,32]. The results were interpreted to indicate that the cell extracts contained an enzyme that catalyzes the reduction of ferredoxin with NADH under the allosteric control of the acetyl-CoA/CoA couple. The reduction of acetyl-CoA was thought to be catalyzed by separate enzymes. Forty years later evidence was presented that these activities all belong to one enzyme, namely the butyryl-CoA dehydrogenase-EtfBC complex (BcdA–EtfBC) catalyzing Reaction (1) (see also Section 4.3) [23,25].

A BcdA–EtfBC complex (Fig. 1) capable of catalyzing Reaction (1) was first purified from \( C. kluyveri \) by using the standard acyl-CoA dehydrogenase assay with butyryl-CoA and ferricenium

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**Table 2**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Abbreviation</th>
<th>Source</th>
<th>Subunits</th>
<th>Prosthetic groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bct/Etf complex</td>
<td>BctA–EtfBC</td>
<td>( C. kluyveri )</td>
<td></td>
<td>(BcdA–EtfBC)₂₅ ( \text{heteroxamer} )</td>
</tr>
<tr>
<td>Butyryl-CoA dehydrogenase</td>
<td>BcdA</td>
<td>( A. fermentans )</td>
<td></td>
<td>4(?) FAD</td>
</tr>
<tr>
<td>Electron transferring flavoprotein</td>
<td>EtfBC</td>
<td>( C. tetanomorphum )</td>
<td></td>
<td>4 FAD</td>
</tr>
<tr>
<td>Electron transferring flavoprotein</td>
<td>EtfBC</td>
<td>( A. fermentans )</td>
<td></td>
<td>2 FAD</td>
</tr>
<tr>
<td>Bifurcating hydrogenase</td>
<td>HydABC</td>
<td>( T. maritima ), acetogenic bacteria</td>
<td></td>
<td>FAD</td>
</tr>
<tr>
<td>Hydrogenase–heterodisulfide reductase</td>
<td>MvhADG–HdrABC</td>
<td>( M. marburgensis ), other methanogens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trans–hydrogenase</td>
<td>NfnAB</td>
<td>( C. kluyveri ), many anaerobic bacteria</td>
<td></td>
<td>FAD, [2Fe–2Fe]</td>
</tr>
<tr>
<td>NAD-ferredoxin reductase</td>
<td>Rnf(RnfABCDEG)</td>
<td>( C. tetanomorphum ), many anaerobic bacteria, ( M. arthascina )</td>
<td></td>
<td>FMN, [2Fe–4Fe]</td>
</tr>
<tr>
<td>Energy converting ([NiFe]) hydrogenase</td>
<td>Ech; EchABCDEF</td>
<td>( E. rubrum ), ( C. thermosphaera ), ( M. arthascina ) and others</td>
<td></td>
<td>FAD, [2Fe–2Fe]</td>
</tr>
</tbody>
</table>

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hexafluorophosphate as electron acceptor. With partially purified fractions the ‘bifurcation assay’ could be applied. The assay comprised crotonyl-CoA, NADH and the enzyme source. NADH oxidation was only observed after addition of catalytic amounts of ferredoxin (Eq. (1)) and the cytoplasmic monomeric [FeFe]hydrogenase from *C. pasteurianum* [33]; the latter was necessary to regenerate oxidized ferredoxin resulting in formation of molecular hydrogen (Eq. (5)).

\[
\text{Fd}_{\text{ox}} + 2\text{NADH} + \text{crotonyl-CoA} \rightarrow \text{Fd}_{\text{red}}^\bullet + 2\text{NAD}^+ + \text{butyryl-CoA} \quad (1)
\]

\[
\text{Fd}_{\text{red}}^\bullet + 2\text{H}^+ \rightarrow \text{Fd}_{\text{ox}} + \text{H}_2
\]

\[
\text{crotonyl-}\text{CoA} + 2\text{NADH} + 2\text{H}^+ \rightarrow \text{butyryl-}\text{CoA} + 2\text{NAD}^+ + \text{H}_2. 
\]

\[
(1 + 5)
\]

Purification of the BcdA-EtfBC complex was performed under strictly anaerobic conditions and in the presence of FAD; otherwise the activity was rapidly lost. The molecular masses of the subunits determined by SDS-PAGE were: \(\alpha\) (Bcd) 41 kDa, \(\beta\) (EtfB) 36 kDa and \(\gamma\) (EtfC) 28 kDa. A photometric scan of an SDS-PAGE run stained with Coomassie brilliant blue yielded a stoichiometry of 1.8:1:1. Gel filtration on Superdex 200 revealed 320 kDa, which matches with 292 kDa, the sum of the subunit molecular masses indicating a quaternary structure of \(\alpha_2\beta_2\gamma_2\) (Fig. 1). N-Terminal sequencing of the three subunits detected the corresponding gene cluster in the *C. kluyveri* genome: *bcdA* (CKL_0455), *etfC* (CKL_0456) and *etfB* (CKL_0457) [25]. The genome contains an additional *bcdA-etfBC* cluster which apparently is not expressed under growth on ethanol and acetate [34].

During the purification of the BcdA-EtfBC complex inactive fractions were detected, which either contained BcdA or EtfBC alone. Hence the complex appeared to dissociate under certain conditions, but whether the recombined fractions regained activity was not tested. Although the amount of FAD in the BcdA-EtfBC complex was not analyzed, it can be assumed that each subunit of the \(\alpha_2\beta_2\gamma_2\) complex contains one molecule of this cofactor. This is based on the structures of acyl-CoA dehydrogenases and EtfBC from *Megasphaera elsdenii*, formerly called *Peptostreptococcus elsdenii*, the only well-studied Etf from a strictly anaerobic bacterium [35,36].

A BcdA-EtfBC complex structurally and functionally almost identical to that from *C. kluyveri* was isolated from *C. tetanomorphum* with subunits as determined by SDS-PAGE: \(\alpha\) (Bcd) 40 kDa, \(\beta\) (EtfB) 36 kDa and \(\gamma\) (EtfC) 28 kDa. The apparent molecular mass of the whole complex determined as 574 kDa by size exclusion chromatography is exactly twice as high as that calculated for the expected \(\alpha_2\beta_2\gamma_2\) composition (288 kDa); hence the complex most likely has an \((\alpha\beta\gamma)_4\) composition [37–39].

In *A. fermentans* (see also Section 4.4) the yellow EtfBC and the green butyryl-CoA dehydrogenase were isolated as separate molecules. Their activities were detected by diaphorase (NADH – iodonitrotetrazolium chloride) and ferricenium assays, respectively. This EtfBC exhibited a FAD-content of 0.5 mol/heterodimer, which increased to 2 mol upon incubation with FAD and removal of the excess by gel filtration (A. M. Mowafy, Z. Li, and W. Buckel, unpublished).

The BcdA-EtfBC complexes from *C. kluyveri* and from *C. tetanomorphum* as well as a 1:1 mixture of Bcd and EtfBC from *A. fermentans* catalyzed the bifurcation reaction according to Eq. \((1 + 5)\). The formed hydrogen was measured by gas chromatography (0.7 H₂/crotonyl-CoA) and the stoichiometry of NADH oxidation was determined as 1.8 ± 0.2 NADH/crotonyl-CoA. Alternatively, oxidized ferredoxin was regenerated with partially purified membrane associated NAD-ferredoxin oxidoreductase from *C. tetanomorphum* also called Rnf (Eq. (6); see Section 3.1.) resulting in Eq. \((1 + 6)\). Due to the presence of detergent in vitro, no \(\Delta\mu\text{Na}^+\) could be observed. In vivo, however, Eq. \((1 + 6)\) represents an anaerobic respiratory chain as will be outlined in Section 4 [38].

\[
\text{Fd}_{\text{red}}^\bullet + \text{NAD}^+ + \text{H}^+ = \text{Fd}_{\text{ox}} + \text{NADH} + (\Delta\mu\text{Na}^+/\text{H}^+); 
\]

\[
(6)
\]

\[
\text{Fd}_{\text{red}}^\bullet + 2\text{NADH} + \text{crotonyl-CoA} \rightarrow \text{Fd}_{\text{red}}^\bullet + 2\text{NAD}^+ + \text{butyryl-CoA}; 
\]

\[
(1)
\]

\[
\text{crotonyl-}\text{CoA} + \text{NADH} + \text{H}^+ = \text{butyryl-}\text{CoA} + \text{NAD}^+ + (\Delta\mu\text{Na}^+/\text{H}^+). 
\]

\[
(1 + 6)
\]

The electron transferring flavoproteins (ETF) are defined as heterodimeric FAD containing proteins mediating electron transport between a dehydrogenase and an ETF-quinone oxidoreductase. The ETFs from clostridia form a special group, which together with an acyl-CoA dehydrogenase catalyze in part the reverse reaction, the reduction of enoyl-CoA by NADH. The mammalian ETF was discovered in 1956 by F. L. Crane and Helmut Beinert as a yellow protein that carries one electron from the usually green acyl-CoA dehydrogenases in the mitochondrial matrix to the inner membrane, where it becomes re-oxidized by ubiquinone (Q) mediated by ETF–Q oxidoreductase (Reactions \((7)\) and \((8)\)) [40]. The ETF-containing mainly tetrameric acyl-CoA dehydrogenases (\(\alpha_4\)) catalyzes the first step of the \(\beta\)-oxidation, the reversible dehydrogenation of acyl-CoA to \((E)\)-2-enoyl-CoA [41]. ETF is a heterodimeric protein (\(\beta\gamma\); human ETF: \(\beta\), 40 kDa; \(\gamma\) 30 kDa) that contains one FAD in the \(\beta\)-subunit and one AMP in the \(\gamma\)-subunit. The crystal structure of the human ETF (Fig. 2) can be subdivided into three domains [42]. Domains I (amino acids 1–205) and II (206–331) stem from the \(\beta\)-subunit, whereas the \(\gamma\)-subunit provides domain III (1–220) and a more than 50 Å long C-terminal arm (221–262) that wraps around domains I and II. The structures of domains I and III are highly related which is already reflected in significant similarities of their primary structures. Presumably, subunits \(\beta\) and \(\gamma\) have evolved from a common ancestor by a gene duplication event. Domains I and III are multiply connected by an extended contact area and constitute a compact core. Domain II is only loosely attached to this core mainly via the mentioned C-terminal arm of the \(\gamma\)-subunit. This architecture of ETF allows conformational changes of domain II relative to the core either induced by NADH or other proteins. FAD is located in a stretched conformation on the surface of domain II with the isoalloxazine ring pointing toward AMP that is embedded into the center of domain III. Hence the distance between AMP and FAD could decrease when domain II moves toward domains I + III. Modeling and cross-linking studies support an interaction of the acyl-CoA dehydrogenase with
ETF between domains II and III, which would be the shortest possible distance for an electron transfer between both FADs [42].

It has been shown that ETF shuttles only one electron from the dehydrogenase to the oxidoreductase (Reactions (7) and (8)). Thus ETF is able to stabilize the red semiquinone anion (ETF•−) [43]. This is structurally supported by the flavodoxin fold of domain II [42] and by a hydrogen bond between 4′-OH of the ribityl side chain and N(1) of the isoalloxazine ring [44].

Acyl–CoA dehydrogenase: Acyl–CoA + 2ETF → Enoyl–CoA + 2ETF•− + 2H+

ETF–Q coxidoreductase: 2ETF•− + 2H+ + Q → 2ETF + QH2. (8)

The human type ETF is not only wide-spread among animals but also common in aerobic bacteria, which thrive on the oxidation of fatty acids [45]. The high sequence identities between the human type ETFs and the acyl-CoA dehydrogenases do not. This corroborates with the presence of the second FAD molecule in EtfBCs of A. fermentans and M. elsdenii, which probably replaces the AMP.

Assuming that all bifurcating EtfBCs contain two FAD, one in each subunit, we propose following hypothetical mechanism, modified after [46] (Fig. 3). NADH reduces γ-FAD (FAD in the γ-subunit) to γ-FADH•−, which due to the unfavorable redox potentials cannot transfer one electron further to ferredoxin. However, β-FAD sitting in the flavodoxin domain of the β-subunit could swing over to the γ-FADH•− and get one electron to become β–FADH•−, which swings back before the next electron can follow. Similar to flavodoxin, the γ-subunit should be able to stabilize the semiquinone anion. The remaining electron on the now formed semiquinone γ-FADH+ is not stabilized by the protein and highly reactive (“red hot”) to immediately reduce ferredoxin (Fd) to Fd− [47]. The stabilized semiquinone anion β–FADH•− transfers the electron further to Bcd where it is stored. Then another NADH enters EtfBC starting a next bifurcation by which Fd− is further reduced to Fd2− and Bcd gets the second electron to convert cytochrome b to Cytc complex (see Section 2.6). An alternative “thermodynamic mechanism” is proposed in Section 2.4.

2.2. HydABC complex coupling reversible ferredoxin reduction with H2 to NAD+ reduction

The second cytoplasmic enzyme complex with an electron bifurcating coupling mechanism was discovered by Schut and Adams in 2009 [26]. They could show that the heterotrimeric [FeFe]hydrogenase from Thermotoga maritima [48], which is rapidly inactivated in the presence of even trace amounts of oxygen, catalyzes the formation of H2 with reduced ferredoxin. Oxidation of 1 mol NADH gave 2 mol H2 (Reaction (2) in the back direction). The reduction of ferredoxin and NAD+ with H2, which is the thermodynamically preferred reaction, was not studied (Reaction (2) in the forward direction) (Fig. 4).

The anaerobically purified hydrogenase from T. maritima was composed of the three subunits HydA (73 kDa), HydB (68 kDa) and HydC (18 kDa) in a 1:1:1 stoichiometry. The holoenzyme showed an apparent molecular mass of 500 kDa at pH 7 and one of 150 kDa at pH 10. The yellow brownish enzyme contained FMN, which was, however, only loosely bound and therefore had to be present in the buffers during the purification procedure; otherwise most of the activity was lost. Some of the lost activity could be restored upon addition of FMN. Surprisingly, FAD also showed a stimulatory effect albeit much less pronounced than FMN. Besides FMN the holoenzyme contained more than 30 Fe per heterotrimer. This value is, within experimental error, the sum of the values predicted from the sequence analysis (see below). Inductively coupled plasma mass spectrometry analysis (ICP) of the holoprotein did not indicate the presence of any other metal other than iron [48].

HydA is predicted by bioinformatic analyses of the encoding gene sequences to harbor the H-cluster and thus the active site interacting with H2. This was confirmed by showing that HydA alone, after dissociation of the complex in urea, can catalyze the reduction of viologen dyes with H2. Besides the H cluster, HydA carries 3 [4Fe–4S] and 2 [2Fe–2S] clusters. HydA shows sequence similarity (43%) to the monomeric [FeFe]hydrogenase I from C. pasteurianum, which catalyzes the formation of H2 with reduced ferredoxin. HydA from T. maritima differs, however, in having a C-terminal extension with a [2Fe–2S] binding site which is lacking in the monomeric enzyme from C. pasteurianum. HydA from T. maritima did not catalyze the reduction of ferredoxin with H2 nor the reverse reaction [48].

HydB shows similarity (70%) to the gene product HndC of the NADP+-reducing [FeFe]hydrogenase from Desulfovibrio fructosovorans and to Nuof of the NADH ubiquinone oxidoreductase from E. coli (60% similarity). Within the sequence there are two highly conserved stretches, one characteristic for NAD+ binding sites and the other for FMN binding sites. The C-terminal part contains Cys motifs that could bind three [4Fe–4S] clusters. At its N-terminal HydB contains four Cys residues that are thought to be involved in binding a [2Fe–2S] cluster [48]. It was not tested whether HydB alone has dehydrogenase activity. HydC shows four Cys residues arranged in a motif with high similarity (58%) to motifs in E. coli Nuo and D. fructosovorans HndA, which are thought to bind a [2Fe–2S] cluster.

From the experimental and bioinformatic analysis a picture of the structure and function of the HydABC complex emerges that is summarized in the cartoon shown in Fig. 4. The binding site for ferredoxin at HydC was chosen arbitrarily. Also, for the second FMN, the one with the question mark, there is no experimental evidence. The number of FMNs bound in the complex could not be determined because the FMN was lost as soon as the FMN was left out from the buffers. We postulate that two FMN should be bound, one being required...
for NADH dehydrogenation and a second one for flavin-based electron-bifurcation.

The three genes encoding HydABC in T. maritima are organized in a cluster hydCBA that is most probably a transcription unit [48]. Clustered genes for proteins with sequence similarity to HydABC are also found in many other anaerobic bacteria namely Clostridium ljungdahlii [49] Acetobacterium woodii [50] and Moorella thermoacetica [51]. Although these gene products have not been characterized, it was shown that cell extracts of the A. woodii [50] and M. thermoacetica [28] catalyze the NAD+ dependent reduction of ferredoxin with H2. Per mol ferredoxin 1 mol NAD+ was reduced (Reaction(2) in the forward direction).

In some anaerobic bacteria, e.g. Thermoanaerobacter tengcongensis the enzyme is composed of four rather than of three different subunits. This is due to the fact that their HydA homolog lacks the C-terminal extension with the [2Fe–2S] cluster and that these hydrogenases instead have a fourth subunit homologous to the C-terminal extension [52]. The electron-bifurcating [FeFe]hydrogenase is not found in archaea which appear to only contain [NiFe]hydrogenase and/or [Fe]hydrogenase [53].

2.3. MvhADG–HdrABC complex coupling the reduction of ferredoxin with H2 to CoM-S-S-CoB reduction

It was long known that in cell extracts of Methanothermobacter species the reduction of metronidazole with H2 is dependent on CoM-S-S-CoB, the heterodisulfide of coenzyme M (CoM-SH) and coenzyme B (CoB-SH) [54]. Metronidazole is spontaneously reduced by reduced ferredoxin and reduced flavodoxin. But it took until a year ago to show that the exergonic reduction of CoM-S-S-CoB with H2 is coupled with the endergonic reduction of ferredoxin with H2 (Reaction (3)). The uphill reduction of ferredoxin with H2 driven by the exergonic reduction of CoM-S-S-CoB with H2 could be directly demonstrated [55].

The cytoplasmic heterohexameric enzyme complex catalyzing Reaction (3) is composed of the six subunits MvhA (53 kDa), MvhG (34 kDa), MvhD (16 kDa), HdrA (72 kDa), HdrC (21 kDa) and HdrB (33 kDa) in equal amounts (Fig. 5). The complex contains about 40 Fe and acid labile sulfur/mol, one Ni, one Zn and probably one FAD which is only loosely bound and which therefore has to be added to the buffers during the purification procedure. Purification has to be performed under strictly anoxic conditions; otherwise the activity is rapidly lost (Fig. 5) [29].

The MvhADG–HdrABC complex can be dissociated into MvhADG and HdrABC. The MvhADG sub-complex catalyzes the reduction of methyl viologen with H2 and the HdrABC sub-complex the reduction of CoM-S-S-CoB with reduced methyl viologen. MvhA harbors the [NiFe] center, which is the site of H2 oxidation, as revealed by sequence analysis. MvhA is synthesized as a pre-protein, but the endopeptidase required for maturation has not yet been found. MvhG is the small subunit of the hydrogenase with a proximal and distal [4Fe–4S] cluster and a medial [3Fe–4S] cluster. MvhD is a [2Fe–2S] iron sulfur protein, which is of interest since in archaeal proteins [2Fe–2S] clusters are an exception. MvhD probably functions as an electronic link between the sub-complexes. In some methanogens the mvhD gene is associated with the hdrABC genes rather than with the mvhAG genes. In Methanosarcina barkeri and in Archaeoglobus fulgidus the mvhD gene is fused to the 3′-end of hdrA [29,53].

HdrA, which harbors the FAD binding site, carries 2 [4Fe–4S] clusters and 4 characteristically spaced cysteines which are conserved in

Fig. 3. Proposed mechanism of flavin based electron bifurcation for the BcdA–EtfBC. The approximate redox potentials $E'$ are given in mV. Black species are oxidized, blue species are reduced by two electrons, and red species carry unpaired electrons. Dotted arrows indicate one (red) or two (blue) electron transfers. Full arrows indicate transformations. For further explanation see text.

Fig. 4. Schematic representation of the structure and function the HydABC complex from Thermotoga maritima. The enzyme is proposed to harbor a second flavin (the one with the question mark). There is no evidence for the proposed pathway of electrons. The [FeFe] center plus [4Fe–4S] cluster colored in orange represent the H-cluster, the active site of the hydrogenase. The site of ferredoxin interaction was chosen arbitrarily.
all HdrA homologs. In HdrA from *Methanococcus* species, one of the four cysteines is replaced by a selenocysteine. HdrA is not the site of heterodisulfide reduction as previously thought. This site has been found in the zinc protein HdrB, which contains two cysteine-rich sequence motifs C_{21–39}CC_{35–36}CCx_{x}x_{x} designated as CCG domains. The C-terminal CCG domain is involved in binding an unusual [4Fe–4S] cluster, and the N-terminal domain is involved in zinc binding. HdrC is a subunit with two [4Fe–4S] clusters. The binding site for ferredoxin at the HdrA subunit was chosen arbitrarily [55, 56].

In the *Methanobacteriales*, *Methanopyrales* and *Methanococcales* the genes encoding the MvhADG–HdrABC complex are organized in three transcription units, *mvhDAG*, *hdrA* and *hdrBC* which are not located adjacent to another. In the *Methanomicrobiales* the *hdr* genes are juxta-positioned; *mvhb* encodes for a polyferredoxin with twelve [4Fe–4S] clusters which partially purifies together with the MchADG–HdrABC complex. The gene *hdrA*, which is highly conserved, is not found only in methanogenic archaea but also in sulfate-reducing archaea and some bacteria which do not contain the genes *mvhAG* and *hdrBC*. This suggests that HdrA in non-methanogens may have an electron bifurcating function within another context than in methanogens. Interesting in this respect is that in most methanogens the *hdr* genes located separately from the *hdrBC* genes consistent with HdrA being used not only in combination with HdrBC [56].

Many members of the *Methanomicrobiales* lack the *mvhAG* genes. It has been proposed that in these hydrogenotrophic methanogens the *F_{2}O_{20}* reducing hydrogenase FrhAG rather than MvhAG forms a functional electron bifurcating complex [29].

### 2.4. The NfnAB complex coupling the reduction of ferredoxin with NADPH to the reduction of NAD^{+}

In 1971 it was reported that cell extracts from *C. kluyveri* catalyzed the reduction of ferredoxin with NADPH only in the presence of NAD^{+} and that concomitant to ferredoxin also NAD^{−} was reduced [57]. The results were interpreted to indicate that cell extracts contained an enzyme that catalyzes the reduction of ferredoxin with NADPH under the allosteric control of the NAD^{+}/NADH couple. The reduction of NAD^{−} with NADPH was thought to be catalyzed by a separate transhydrogenase. Forty years later evidence was presented that these activities all belong to one enzyme, namely the NADH-dependent reduced ferredoxin: NADP oxidoreductase, abbreviated Nfn, Reaction (4) [27].

Nfn is a heterodimeric enzyme composed of the subunits NfnA (30 kDa) and NfnB (50 kDa) present in a 1:1 stoichiometry (Fig. 6). NfnA shows sequence similarities to ferredoxin-NADP^{+} reductase from plants and has binding sites for NAD(P)H, FAD and one [2Fe–2S] cluster. Most likely this subunit interacts with ferredoxin. NfnB shows sequence similarities to the β-subunit of the NADP^{+}-dependent glutamate synthase and also has binding sites for NAD(P)H, FAD and two [4Fe–4S] clusters. During purification, the loosely bound FAD is lost with concomitant loss of activity. The activity could be partially restored by addition of FAD rather than FMN (Fig. 6).

The NfnAB complex dissociates relatively easily into NfnA and NfnB which after separation can therefore be tested separately. The UV–visible spectrum revealed that both subunits are iron–sulfur flavoproteins. Neither NfnA nor NfnB alone catalyzed Reaction (4), but when they were mixed in a 1:1 ratio, the resulting complex was active. NfnA catalyzed the reduction of benzyl viologen with NADPH (0.05 U/mg) and with NADH (0.01 U/mg), whereas NfnB catalyzed the reduction of benzyl viologen with NADPH (2 U/mg) and with NADH (0.07 U/mg). These activities did not change in the presence of NAD^{+} or NADP^{−}. Unfortunately, from these results it cannot be deduced which subunit carries the NAD^{−} and which the NADP^{−} binding site.

The genes *nfnA* and *nfnB* in *C. kluyveri* are juxta-positioned and form a transcription unit. They can be heterologously expressed in *E. coli* carrying extra genes for the synthesis of iron–sulfur clusters. When expressed together, the resulting heterodimeric enzyme catalyzed Reaction (4). The heterologously produced enzyme contained only 6 to 7 iron per heterodimer. The iron content increased up to 10 after iron–sulfur cluster reconstitution. Concomitantly, the specific activity almost doubled. This finding is consistent with the presence of one [2Fe–2S] cluster and two [4Fe–4S] clusters in the heterodimer.

Genome analyses revealed the nfnAB genes to be present in many *Clostridiales*, an exception being *C. acetobutylicum*. Interestingly, in *C. ljungdahlii* the *nfnA* and *nfnB* genes are fused. They are also present in many other *Firmicutes* of the order *Clostridiales*, namely *Eubacterium*, *Thermoclostrum*, *Carboxidothermus*, *Desulfotomaculum* and *Moorella*. NfnAB from *M. thermoacetica* has been characterized and shown to have properties very similar to NfnAB from *C. kluyveri* [28].

The NfnAB genes are also found outside the group of the *Firmicutes* e.g. in *Bacteroides*, *Thermotoga*, *Pyrococcus*, *Thermococcus* and *Methanosarcina*. The NfnAB genes from *T. maritima* have been expressed in *E. coli* and the product has been shown to catalyze the NADH dependent reduction of NADP^{+} with reduced ferredoxin (Reaction (4), H. Huang and R.K. Thauer, unpublished).

For NfnAB a mechanism of flavin based electron bifurcation has been proposed assuming that the FAD involved in the bifurcation is bound in the flavoprotein differently tight in its oxidized, quinone (FAD), one electron reduced, semiquinone (FADH^{+}) and two electrons reduced, hydroquinone states (FADH_{2}) [27]: E^{O}_{r} of the FAD/FADH couple in solution is −205 mV (Table 1). The redox potential E^{O}_{r} is lower when FAD in the flavoprotein is bound more tightly than FADH^{+}. The redox potential of the FADH^{+}/FADH^{2} couple is more negative than that of the FADH^{+}/FADH couple when FADH^{+} binds tighter than FADH^{2}. In the case of n = 2 the redox potential changes by about 30 mV per factor of 10 difference in binding constants and in the case of n = 1 it changes by about 60 mV per factor of 10. It has been pointed out by Nitschke and Russell, however, that this is probably not how electron bifurcations work [47]. Their arguments are.

"But why should the fully reduced flavin need the second oxidation
step to form the first one? It is already in the regime of separated 1-electron transfers and will possibly reduce ferredoxin but, even much more readily, will reduce the high potential acceptor due to the much stronger driving force for electron transfer to the latter and then just remain as a flavosemiquinone.

The “thermodynamic model” proposed by Wang et al. [27] differs from the proposed kinetic model described in Fig. 3 in the sequence of reactions. Whereas in the thermodynamic model ferredoxin is reduced by FADH$^-$ like in flavodoxins, in the kinetic model ferredoxin is reduced by the highly reactive unstable FADH$^+$ radical generated by the one electron oxidation of FADH$^+$. To discriminate between the two mechanisms, Nitschke and Russell predict that equilibrium redox titration of the flavin should demonstrate an n = 2 Nernst transition and a concomitant absence of a stable flavosemiquinone species [47].

2.5. Other ferredoxin-dependent enzyme complexes possibly catalyzing flavin-based electron bifurcation reactions

It has been postulated that the reduction of methylene-H$_4$F to methyl-H$_4$F (E$_c$ = $-$200 mV) in the acetogenic bacteria is coupled with the reduction of ferredoxin via flavin-based electron bifurcation [49]. Information on the methylene-H$_4$F reductase is available for A. woodii and M. thermotoga. In A. woodii the gene probably encoding for methylene-H$_4$F reductase is found in a transcription unit of three genes that – on the protein level – show sequence similarities to the methylene-H$_4$F reductase from E. coli, to zinc finger proteins and to a NAD$^+$ binding flavoprotein, respectively [50]. In cell extracts methylene-H$_4$F is reduced specifically by NADH, the presence of ferredoxin not being required [58]. In M. thermotoga the gene with sequence similarity to methylene-H$_4$F reductase from E. coli is located in a gene cluster together with genes for a zinc finger protein and four proteins homologous to MvhB, HdrA, HdrB and HdrC that are found in the electron bifurcating MvhHADG–HdrABC complex catalyzing Reaction (3) [28]. Cell extracts of M. thermotoga catalyze the reduction of methylene-H$_4$F only with reduced viologen dyes rather than with reduced ferredoxin, NADH or NADPH, neither when tested alone or in combinations.

Another candidate for flavin-based electron bifurcation is the ATP-independent benzoyl-CoA reductase from strictly anaerobic bacteria of the genera Geobacter and Syntrophus that are energy limited [59,60]. Benzoyl-CoA is the key intermediate in the anaerobic degradation of aromatic compounds. The tungsten iron–sulfur flavoprotein from G. metallireducens catalyzes the reduction of benzoyl-CoA to cyclohexa-1,5-dienoyl-1-carboxyl-CoA (dienoyl-CoA) most likely with reduced ferredoxin as electron donor although this has not yet been ascertained. Thermodynamically this is an uphill reaction since the redox potential E°$^{\text{red}}$ of the dienoyl-CoA/benzoyl-CoA couple is $-$622 mV [59]. The uphill reduction of benzoyl-CoA to dienoyl-CoA with reduced ferredoxin must therefore somehow be coupled with a downhill oxidation of reduced ferredoxin most probably with NAD$^+$. The finding that the enzyme complex is composed of 8 different subunits, four of which show sequence similarities to the subunits MvhB, HdrA, HdrB and HdrC of the electron bifurcating MvhHADG–HdrABC complex from methanogenic archaea (Section 2.2), supports this proposal. Anaerobes that are not energy limited as the denitrifier Thauera aromatica use an ATP-dependent benzoyl-CoA reductase [22] which is not phylogenetically related to the ATP-independent enzymes.

More than 40 years ago it was found that cell extracts of Clostridium acid-urici catalyze the reduction of CO$_2$ to formate only in the presence of reduced ferredoxin and NADH [61]. It is thus possible that the organism contains an enzyme that couples the exergonic reduction of NAD$^+$ by formate with the oxidation of ferredoxin by formate, similar to the HydABC complex from T. maritima in which the hydrogenase couples the exergonic reduction of NAD$^+$ by H$_2$ with the reduction of ferredoxin by H$_2$ [26].

Comparative genome analysis of energy metabolism in sulfate reducing bacteria and archaea has revealed several transcription units for cytoplasmic enzyme complexes containing homologs of HdrA, HdrB and HdrC. It has been speculated that these complexes catalyze electron bifurcating reactions, especially with adenosine phosphosulfate as high potential electron acceptor (E°$^{\text{red}}$ ca. $-$100 mV) [62,63].

2.6. Comparison of flavin-based electron bifurcation with ubiquinone-based electron bifurcation

Electron bifurcation was first proposed by Peter Mitchell in 1976 to explain the reduction of the low potential cytochrome $b_5$ in mitochondria by addition of the strong oxidant ferricyanide [64]. This ingenious idea resulted in the establishment of the Q-cycle, in which the oxidation of ubiquinol (QH$_2$) by cytochrome c causes the translocation of two protons per electron across the inner mitochondrial membrane [65]. An essential feature of the cycle is the two binding sites for QH$_2$, the Qo and Qi sites and that QH$_2$ at the Qo-site is oxidized simultaneously by the Rieske [2Fe–2S] cluster in the iron sulfur protein (ISP) and by cytochrome $b_5$ (cyt $b_5$), each of which removes one electron and the two protons are released to the outside (Fig. 7). This process has been called bifurcation, because the two electrons from QH$_2$ are separated to different energy levels like the tines of a fork (Latin furca = fork). One electron goes to the iron sulfur protein with a higher redox potential (E°$^{\text{red}}$ = +320 mV) and the other to cyt $b_5$ with a lower redox potential (E°$^{\text{red}}$ = $-$90 mV) than QH$_2$ (E°$^{\text{red}}$ = +90 mV). In the proton motive Q cycle, the one-electron oxidation of QH$_2$ by the Rieske cluster triggers the reduction of cyt $b_5$. Then the Rieske cluster in the iron sulfur protein flips over to enable the transfer of the electron to cyt $c_1$ (E°$^{\text{red}}$ = +263 mV) and further to the final acceptor cyt c (E°$^{\text{red}}$ = +230 mV) [66]. The electron in cyt $b_5$ moves via cyt $b_6$ (E°$^{\text{red}}$ = +50 mV) to the Qo-site where it reduces the bound Q to a stabilized semiquinone. The bifurcation is repeated with a second QH$_2$ at the Qo-site yielding another reduced cyt c and a fully reduced QH$_2$ at the Qo-site, whereby two protons are released again to the cytoplasm and two are taken up from the matrix. In summary one QH$_2$ is oxidized to afford two reduced cyt c, whereby four protons are translocated to the cytoplasm and two are taken up from the matrix. The other two protons stem also from the matrix during reduction of Q to QH$_2$, by NADH + H$^+$, succinate or two reduced one-electron transferring flavoproteins (ETF$^{\text{red}}$) [67–69].
3. Membrane-associated enzyme complexes coupling exergonic ferredoxin oxidation reactions with vectorial proton/Na\(^+\) translocation

The generation of reduced ferredoxin in the electron bifurcating Reactions (1)–(4) conserves part of the free energy change associated with these reactions in a redox potential \(E = -500 \text{ mV}\) which is more negative than that of the NAD\(^+\)/NADH couple \(E = -280 \text{ mV}\) and that of the H\(^+\)/H\(_2\) couple (dependent on pH\(_2\) between -300 mV and -414 mV) (see Introduction). The redox potential difference between ferredoxin and NAD\(^+\) (\(\Delta E = 220 \text{ mV}\)) is sufficient to allow the build-up of an electrochemical proton or sodium ion potential (\(\Delta \mu_{\text{H}^+}/\mu_{\text{Na}^+}\)) that in turn could be used to drive the phosphorylation of ADP via a membrane associated F\(_{\text{ox}}\)F\(_{\text{1}}\) or A\(_{\text{23}}\)A\(_{\text{1}}\)ATP synthase complex. The same is true for the redox potential difference between ferredoxin and protons if pH\(_2\) is kept low by H\(_2\)-consuming microorganisms, which is the same is true for the redox potential difference between ferredoxin and protons if pH\(_2\) is kept low by H\(_2\)-consuming microorganisms, which is the case in most anaerobic habitats. And indeed, genome sequencing revealed that many anaerobes contain the genes for a membrane associated RnfABCDEFG complex [71] and/or a membrane associated, energy converting [NiFe]hydrogenase complex [72] catalyzing the reversible oxidation of reduced ferredoxin with NAD\(^+\) (Reaction (6)) and H\(^+\) (Reaction (10)), respectively.

\[
\begin{align*}
\text{Fd}_{\text{red}}^{-} + \text{H}^+ + \text{NAD}^+ &\rightleftharpoons \text{Fd}_{\text{ox}} + \text{NADH} + \Delta \mu_{\text{H}^+}/\text{Na}^+ \quad (6) \\
\text{Fd}_{\text{red}}^{-} + 2\text{H}^+ &\rightleftharpoons \text{Fd}_{\text{ox}} + \text{H}_2 + \Delta \mu_{\text{H}^+}/\text{Na}^+ \quad (10)
\end{align*}
\]

3.1. RnfA-G complex coupling ferredoxin oxidation by NAD\(^+\) with proton/Na\(^+\) translocation

The phototrophic bacterium *Rhodobacter capsulatus* contains a cluster of six genes designated as *rfnABCDEFG* that is thought to be involved in electron transfer to nitrogenase (*Rhodobacter* nitrogen fixation = Rnf) [73,74]. Homologous clusters, which code for such a membrane bound NAD\(^+\)-ferredoxin oxidoreductase, are found in over a hundred aerobic and anaerobic bacteria but only in two archaea [71]. Membrane vesicles of *C. tetanomorphum* catalyze the oxidation of NADH by hexacyanoferrate(III) (ferricyanide) at a rate of 26 Um\(^-1\) protein and the reduction of NAD\(^+\) by ferredoxin [reduced with Ti(III) citrate] with 1.5 Um\(^-1\) protein. The enzyme has been solubilized from the membrane fraction and purified to apparent homogeneity with specific activities of 400–1000 Um\(^-1\) protein (ferricyanide assay). The protein consists of the predicted six different subunits RnfABCDEFG, which are separated by SDS-PAGE: C 49 kDa (47 kDa predicted from the gene), B 36 (30) kDa, D 33 (33) kDa, G 26 (20) kDa, A 23 (21) kDa, and E 22 (21) kDa. Sequence analysis of RnfC shows no interaction of the protein with the membrane but indicates the presence of two ferredoxin-like [4Fe-4S] clusters as well as FMN and NADH binding sites. In contrast, the sequences of RnfA, D and E reveal 6, 7 and 5 transmembrane α-helices, respectively, whereas RnfB and G appear to be anchored in the membrane by one α-helix each. RnfD and G contain covalently bound riboflavin-5′-phosphate (FMN), which is linked to the protein via a phosphodiester bond. There is also non-covalently bound FMN and riboflavin present in the enzyme complex. The determined 25±1 iron atoms/hexamer match with the predicted six ferredoxin-like [4Fe-4S] clusters, two in subunit C, and four in subunit B, also called polyferredoxin [38,75–77] (Fig. 8).

Interestingly, four of the six RNF-subunits are related to four of the six subunits of the Na\(^+\)-translocating NADH-quinone oxidoreductase (Na\(^+\)-NQR) from *Vibrio cholerae* and other *Vibrio* species (Fig. 8); for a review see [78], RnfC shares sequence identities with NqrF, RnfD with NqrB, RnfC with NqrC and RnfE with NqrD. Furthermore, NqrB (RnfD) and NqrC (RnfG) also contain covalently bound FMN. Non-covalently bound FAD + [2Fe-2S] cluster, ubiquinone-8 (Q) and riboflavin (RF) are present in subunits F, A and B, respectively [79–81]. The electrons are proposed to flow from NADH via subunits F, C, B and A to the external ubiquinone, which induce conformational changes in B triggering E and D to translocate Na\(^+\).

![Fig. 7. Schematic presentation of the Q-cycle in complex III or cytochrome b\(_5\) of the mitochondrial respiratory chain. Q\(_i\) and Q\(_o\), ubiquinone at the inner and outer sides of the inner mitochondrial membrane; b\(_h\) and b\(_l\), cytochrome b with lower and higher redox potentials; FeS, Rieske [2Fe-2Fe] cluster in the iron sulfur protein; c and c\(_1\), cytochrome c and c\(_1\). Adapted from [70].](image-url)
A similar path of the electron, but in the opposite direction, could be constructed in Rnf: ferredoxin → RnfB → RnfD (NqrB) → RnfG (NqrC) → RnfC (NqrF) → NADH, which induces a conformational change in RnfD triggering RnfE (NqrD) and RnfA to generate an electrochemical Na⁺-gradient (ΔμNa⁺, Fig. 8). It should be noted that NqrA and NqrD are not related to RnfB and RnfA, which is certainly due to the different electron donor in Rnf (ferredoxin). Most likely, the electron flow in Rnf is reversible as proposed for nitrogen fixation in R. capsulatus to enable a NADH-dependent reduction of ferredoxin driven by ΔμH⁺/Na⁺ [73].

Recently, it has been demonstrated that Rnf-containing membrane vesicles from A. woodii indeed couple the reduction of Na⁺/H₂ by reduced ferredoxin with the formation of ΔμNa⁺ that can be used for ATP-synthesis or transport processes [82] as proposed earlier for Clostridium tetani [83]. Similar experiments have been performed with vesicles from C. tetanomorphum (E. Jayamani, J. Steuber, E. Biegel, V. Müller and W. Buckel, unpublished). Notably, however, in both systems the Na⁺ transport exhibited an apparent Kₘ = 2.5 mM Na⁺, whereas Na⁺/H₂ reduction by ferredoxin was not stimulated by Na⁺ in the range of 0.1–100 mM. In contrast, the scalar rates of established Na⁺-pumps, e.g. Na⁺–NQR [84], glutacnyl-CoA decarboxylase [85,86] and diphosphatase [87], are dependent on Na⁺, each with Kᵥ values for Na⁺ in the lower mM range. Furthermore, with Rnf the measured Na⁺ transport rates are more than 1000-times slower [82] than the scalar rate of the reduction of NADH by ferredoxin catalyzed by membrane vesicles from C. tetanomorphum (see above). Hence, the question whether Rnf is a real Na⁺-pump or a H⁺-pump that just fortuitously translocates Na⁺ remains to be established.

3.2. Energy converting [NiFe]hydrogenase complexes coupling ferredoxin oxidation with proton/Na⁺ translocation

Energy converting [NiFe]hydrogenases are membrane associated and catalyze the reversible reduction of ferredoxin with H₂ driven by the proton or sodium ion motive force, Reaction (10) [72]. They are found in some H₂-forming and H₂-utilizing anaerobic bacteria and archaea. Most convincing is the energy-converting function of the [NiFe]hydrogenase in the Gram-negative Rhodospirillum rubrum and Rubrivivax gelatinosus as well as the Gram-positive Carboxidothermus hydrogenoformans. These anaerobic bacteria can grow chemolithoautotrophically on CO, with CO₂ and H₂ being the only catabolic end products. The energy metabolism, which only involves a cytoplasmic nickel-containing carbon monox- ide dehydrogenase, a cytoplasmic polyferredoxin (electron transfer protein) and a membrane associated [NiFe]hydrogenase complex is coupled with chemiosmotic energy conservation as evidenced by growth and uncoupling experiments [88].

The membrane-associated energy-converting [NiFe]hydrogenase complex involved in CO conversion to CO₂ and H₂ is composed of six different subunits of which two are integral membrane proteins (the larger one most probably involved in cation translocation) and four are peripheral membrane proteins oriented toward the cytoplasm. Of the four peripheral proteins, one carries the [NiFe]hydrogenase active site; one harbors 1 [4Fe–4S] cluster most proximal to the [NiFe] center and one harbors 2 [4Fe–4S] clusters transporting the electrons to the ferredoxin binding site. The fourth peripheral protein is without a prosthetic group. The six subunits show sequence similarities to six of the core subunits of the [NiFe]hydrogenase from E. coli. In complex I, the subunit NuoD homologous to the [NiFe] center carrying subunit lacks the N-terminal and C-terminal CxxC motifs for [NiFe] center binding [53] (Fig. 9).

An energy converting [NiFe]hydrogenase with six subunits is also found in microorganisms not growing on CO such as E. coli, M. barkeri and M. mazei [88]. In E. coli the hydrogenase is part of the formate hydrogen lyase complex catalyzing the conversion of formate to CO₂ and H₂. In the two methanogens the six-subunit [NiFe]hydrogenase differs from those in the CO hydrogen lyase complex and the formate hydrogen lyase complex only in not forming a tight complex with its ferredoxin and functionally associated oxidoreductase. Methanogens lack complex formation because the reduced ferredoxin, generated by the energy converting hydrogenase, is used in electron transfer to more than one oxidoreductase. A two [4Fe–4S] cluster-containing ferredoxin (6 kDa) from M. barkeri, which is most probably the ferredoxin reduced by H₂ via the hydrogenase, has been characterized [53].

Besides the six subunit energy converting [NiFe]hydrogenases there are energy converting hydrogenases, abbreviated as Mbh, which contain, in addition to the six core subunits, up to 8 integral membrane proteins as additional subunits. The MbhA-N hydrogenase is found in some members of the Methanomicrobiales (Methanospirillum hungatei and Methanocorpusculum labreanum) and in Pyrococcus furiosus, from which the enzyme complex was purified and characterized [89,90].

In most hydrogenotrophic methanogens there is a third type of energy-converting [NiFe]hydrogenase complex, EhaA–T and EhbA–Q, with up to 20 subunits of which only four show sequence similarity to subunits of the [NiFe]hydrogenase from R. rubrum. These are the two conserved integral membrane proteins and the two proteins harboring the [NiFe] center and the [4Fe–4S] cluster, respectively. A core subunit with 2 [4Fe–4S] clusters appears to be lacking. Instead, there is a 6 [4Fe–4S] cluster polyferredoxin (EhaP) or a 10 [4Fe–4S] cluster polyferredoxin (EhaQ) in the EhaA–T complex as well as a non-conserved 2 [4Fe–4S] cluster ferredoxin (EhbL) and a 14 [4Fe–4S] cluster polyferredoxin in the EhbA–Q complex. Of the additional other subunits most are non-conserved integral membrane proteins [53]. The standard [NiFe]hydrogenase complexes composed of the 6 core subunits probably all translocate protons, whereas the larger [NiFe]hydrogenase complexes MbhA–N, EhaA–T and EhbA–Q most probably translocate sodium ions [53].

4. Anaerobes conserving energy via electron bifurcating ferredoxin reduction and/or proton/Na⁺ translocating ferredoxin oxidation

Comparisons of many energy metabolisms of aerobic and anaero- bic chemotrophic organisms have revealed that between 60 and 80 kJ is required in a living cell to synthesize ATP from ADP and inorganic phosphate [11]. The higher value is more characteristic for aerobes and the lower value for anaerobes. Under equilibrium conditions only about 50 kJ/mol ATP is required. During energy conservation, the difference is dissipated as heat (Fig. 10).

The beauty of the relatively constant amount of energy required for the synthesis of ATP is the possibility to predict the numbers of ATP synthesized, provided the free energy change associated with the number of ATP known to be generated via substrate level phosphorylation (SLP) and electron transport phosphorylation (ETP). In the energy metabolism of some anaerobes, however, there was no agreement indicating that all sites of energy conservation (ETP) in the energy metabolism were not yet known. The finding of energy conservation via electron bifurcating ferredoxin reduc- tion and of proton/Na⁺ translocating ferredoxin oxidation in many anaerobes has closed this gap as will be shown in the following examples (Fig. 10).

4.1. Glucose fermentation by Clostridium pasteurianum

C. pasteurianum ferments 1.5 glucose to 1 acetate, 1 butyrate, 2 CO₂ and 4 H₂ (Fig. 11). H₂ bubbles out of the cultures indicating that the H₂ partial pressure is near 100 kPa (1 bar) and that the redox potential of the H⁺/H₂ couple is at least — 400 mV. Glucose is transported into the
cells via a phosphoenolpyruvate (PEP) transferase system \[91,92\] and the oxidation to 2 pyruvate proceeds via the Embden–Meyerhof pathway, generating 3 NADH and 3 ATP per 1.5 mol glucose. The 3 pyruvates are further oxidized to 3 acetyl-CoA with 3 ferredoxin as electron acceptor. One acetyl-CoA gives rise to 1 acetate and 1 ATP and the other 2 acetyl-CoA are reduced to butyrate concomitant with the formation of 1 ATP. The organism contains a ferredoxin-dependent monomeric [FeFe]hydrogenase \[93\], which catalyzes the re-oxidation of the ferredoxin with the formation of H2. Since 4 rather than 3 mol H2 is formed per 1.5 mol glucose \[11\], there must be a route from NADH to ferredoxin. Considering that the redox potential of the NAD+/NADH couple in the cells is \(-280\) mV and that of the H+/H2 couple is near \(-400\) mV, ferredoxin reduction with NADH is only possible if coupled to the exergonic crotonyl-CoA reduction with NADH. If the two reactions were not coupled, all the NADH generated in the Embden–Meyerhof pathway would have to be re-oxidized via butyric acid formation from acetyl-CoA resulting in a fermentation of 1.5 glucose to 1.5 butyrate, 3 CO2, and 3 H2. Thereby 4.5 mol ATP would be generated rather than 5.0 mol ATP predicted from Fig. 11 (75 kJ/ATP). Thus flavin-based electron bifurcation enhances energy conservation by 11%; in the following examples the contribution is much higher. The alternative pathway of reduced ferredoxin oxidation by NAD+ is excluded, because the membrane fraction of \( \text{C. pasteurianum} \) does not contain ferredoxin: NAD+ oxidoreductase (Rnf) activity. Consistent with that finding, the genome sequence of the closely related \( \text{Clostridium acetobutylicum} \) lacks genes for the RnfA–G complex \[94\].

**4.2. Glucose fermentation by \( \text{Thermotoga maritima} \) and \( \text{Pyrococcus furiosus} \)**

The extremophilic bacterium \( \text{T. maritima} \) thrives by fermenting 1 glucose to 2 acetate, 2 H+, 2 CO2 and 4 H2 at 90 °C. At this temperature
the free energy change of the reaction \(\Delta G^o' = -250 \text{ kJ/mol} \) is about 35 kJ/mol more exergonic than at 25 °C \(\Delta G^o = -215 \text{ kJ/mol} \) allowing for the synthesis of 4 mol ATP per mol glucose from which 1 ATP has to be subtracted for glucose import mediated by a specific ABC transporter [98]. This results in an efficiency of 83 kJ/ATP even at H2 partial pressures near 100 kPa. Glucose is fermented via the Emden–Meyerhof pathway regenerating 2 NADH and via pyruvate:ferredoxin oxidoreductase regenerating 2 reduced ferredoxin [99] (Fig. 12). The formation of 4 H2 indicates that all reducing equivalents from NADH escape as hydrogen. To make this thermodynamically possible, the endergonic reduction of protons to H2 with NADH is coupled to the exergonic reduction of protons to H2 with reduced ferredoxin as catalyzed by the electron bifurcating HydABC complex [26] (Section 2.2.).

The same equation for glucose fermentation has been observed with the archaeon \(P. \text{ furiosus} \) thriving at 100 °C, though the mechanism of energy conservation differs in many respects (Fig. 12). In this organism the phosphorylating NAD+-dependent glycerolaldehyde-3-phosphate dehydrogenase is replaced by a ferredoxin-dependent non-phosphorylating enzyme [19,100,101]. Thus only 2 ATP/glucose are obtained by SLP. However, 4 reduced ferredoxin generate 4 H2 mediated by an energy conserving hydrogenase (Mbh) [90] (see Section 3.2.) resulting in 4×2 ΔμNa+ equal to 2 ATP [102]. By subtracting 1 ATP for glucose import via an ABC transporter [103], the thermodynamic efficiencies of ATP synthesis in the bacterium and the archaeon are identical. The \(P. \text{ furiosus} \) fermentation also demonstrates that reduced ferredoxin can be regarded as an ‘energy rich’ compound due to its more negative redox potential as compared to that of the H+/H2 couple. Whereas, thioesters and anhydrides are ‘energy rich’ because of their ΔG° of hydrolysis \(\approx -30 \text{ kJ/mol} \) (\(\approx \Delta Ge'\) of ATP-hydrolysis), reduced ferredoxin with an \(E' = -500 \text{ mV} \) is ‘energy rich’ because of its physiologically more negative ΔE° \(\approx -nF \Delta E'\) as compared to \(E' > -414 \text{ mV} \) of the H+/H2 couple and to \(E' > -320 \text{ mV} \) of the NAD+/NADH couple.

### 4.3 Ethanol-acetate fermentation of \(C. \text{ kluwyeri}\)

\(C. \text{ kluwyeri}\) grows on ethanol, acetate as energy source and acetate and CO2 as carbon source. Fermentation products are butyrate, caproate, and one proton per 2 H2, whereby 1 ATP is formed via SLP [104]. The cells contain a ferredoxin-dependent monomeric [FeFe]hydrogenase for the generation of H2 and a pyruvate:ferredoxin oxidoreductase for the synthesis of pyruvate from acetyl-CoA and CO2 [34]. Until recently it was not known how the 2.5 mol ATP (72 kJ/ATP) is generated that is predicted from the free energy change associated with the fermentation \(\Delta G^o' = -180 \text{ kJ/mol} \text{ H}^+ \) (Fig. 13A). It was also not known how the reduced ferredoxin required for the two reactions is regenerated. The finding that in \(C. \text{ kluwyeri}\) the exergonic reduction of crotonyl-CoA to butyryl-CoA is coupled with the endergonic reduction of ferredoxin with NADH via the BcdA-EtTBC complex (Section 2.1) and that the organism contains an RnfA–G complex (Section 3.1.) has solved these questions [25,34].

In the ethanol acetate fermentation the number of substrates exceeds the number of products. Therefore, when the concentration decreases to 1 mM, the free energy change decreases to \(-77 \text{ kJ/mol} \text{ H}^+ \) that can only support the synthesis of 1 ATP (Fig. 13B). (In the calculation the concentrations of water, 55.5 M, and protons, pH 7.0, do not change and therefore are not considered.) The switch from 2.5 ATP to 1 ATP is realized by two additional enzymes, namely the electron bifurcating NfnAB complex and a NADP+- dependent β-hydroxybutyryl-CoA dehydrogenase [34]. By tuning the activities of these two enzymes, \(C. \text{ kluwyeri}\) can maintain the efficiency of ATP synthesis constant when the free energy available in the environment changes. Whether regulation is on the transcriptional level or on the posttranslational level is not yet known [27].

### 4.4 Glutamate fermentation by \(C. \text{ tetanomorphum} \) and \(A. \text{ fermentans}\)

Glutamate is fermented in several anaerobic bacteria of the phyla \(Firmicutes\) and \(Fusobacteria\) by two different pathways to identical products (Fig. 14): ammonia, CO2, acetate, butyrate and hydrogen, \(\Delta G^o' = -317 \text{ kJ/mol} \text{ H2} \) [105,106]. According to the balanced fermentation equation, fewer molecules are consumed than produced (Fig. 14). Therefore, the free energy \(\Delta G\) becomes more negative at lower concentrations (see Section 4.3). Assuming the physiological concentrations of glutamate, acetate, butyrate and ammonia to be 1 mM each, \(\Delta G\) decreases to \(-450 \text{ kJ/mol} \text{ H2}\).

\(C. \text{ tetanomorphum}\) and \(C. \text{ tetani} \) ferment glutamate via 3-methylaspartate to ammonia, acetate and pyruvate mediated by the coenzyme B12-dependent glutamate mutase [107] and three further enzymes (Fig. 14). Five pyruvates are then oxidized to 5 acetyl-CoA and 5 reduced ferredoxin (\(F_{\text{red}}\)) that are converted together with an additional \(F_{\text{red}}\) to 6 NADH mediated by Rnf yielding 12 ΔμNa+. Four acetyl-CoA are reduced by 6 NADH to 2 butyryl-CoA, in whose syntheses flavin-based electron bifurcation is involved resulting in additional 2 \(F_{\text{red}}\), one of which reduces protons to H2. Three ATP are formed by SLP from 1 acetyl-CoA and 2 butyryl-CoA [23]. For the import of 5 glutamate probably 5 ΔμNa+ are consumed [83], leaving 7 ΔμNa+ for the synthesis of further 1.75 ATP, all together 0.95 ATP/glutamate with the high efficiency of 67 kJ/mol ATP under standard conditions. At lower concentrations the efficiency decreases; at the assumed 1 mM physiological concentrations (see above) it reaches 96 kJ/mol ATP.

The pathway via 2-hydroxylutarate in \(A. \text{ fermentans}\) (Fig. 14) transforms 5 glutamate to 5 ammonia and 5 \(\times (R)-\)2-hydroxyglutarate that are activated by 5 × acetyl-CoA to 5 \(\times 2\)-hydroxyglutaryl-CoA. Subsequent dehydration affords 5 × glutaryl-CoA that are decarboxylated to 5 × crotonyl-CoA catalyzed by the biotin-containing membrane enzyme glutaryl-CoA decarboxylase (Gcd) that converts the free energy of decarboxylation to an electrochemical Na+ gradient resulting in 10 ΔμNa+ [85]. Three of 5 crotonyl-CoA are oxidized to 6 acetyl-CoA with the formation of 3 NADH, which together with a fourth NADH are consumed in the reduction of the remaining 2 crotonyl-CoA to 2 butyryl-CoA concomitant with electron bifurcation leading to 2 \(F_{\text{red}}\). One \(F_{\text{red}}\) gives rise to H2, and the other recycles the fourth NADH via Rnf adding 2 ΔμNa+ to the conserved energy [23]. If 5 ΔμNa+ for sodium-glutamate symport [108] are subtracted from 12 ΔμNa+, the net yield of ATP is identical to that via 3-methylaspartate.

As indicated above the thermodynamic efficiency of ATP synthesis is only about 100 kJ/ATP when the two organisms ferment glutamate at 1 mM substrate and product concentrations using the pathways shown in Fig. 14. This very low efficiency is not very likely. It can therefore be predicted that the two organisms increase their efficiency by changing the pathways such that the H2 production is increased.

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**Fig. 10.** ATP as central energy carrier in all living cells. Under standard reversible conditions 32 kJ/mol is required for the synthesis of 1 mol ATP from ADP and inorganic phosphate. Under physiological conditions (concentrations of ATP, ADP and \(P_i = 1 \text{ mM rather than 1 M} \)) the value is \(-50 \text{ kJ/mol} \). Under the irreversible conditions in living cells, it generally takes between 60 and 80 kJ to drive the phosphorylation of ADP.
when the concentrations of substrate and products are low as they are in the natural environments where these organisms thrive. That such a change indeed may occur has been demonstrated for *C. kluyveri* in the previous chapter (Section 4.3).

Via the methylaspartate pathway [85,86] the fermentation of glutamate could proceed to acetate and H₂ without butyrate production [109]: Glutamate $\rightarrow$ +2 H₂O $\rightarrow$ NH₄⁺ + CO₂ + 2 Acetate⁻ + H₂; $\Delta G'' = -45$ kJ/mol glutamate; $\Delta G'$ (1 mM) = $-113$ kJ/mol glutamate.

**Fig. 11.** Glucose fermentation by *Clostridium pasteurianum*. The FiFo ATPase functions in the direction of ATP hydrolysis rather than ATP synthesis [95,96]. PEP, phosphoenolpyruvate; Fd, ferredoxin; B/E, BcdA–EtfBC complex (yellow spot).

**Fig. 12.** Glucose fermentation in the hyperthermophilic bacterium *Thermotoga maritima* and the hyperthermophilic archaeon *Pyrococcus furiosus*. In *T. maritima* the A₅A₅ ATPase functions in the direction of ATP hydrolysis rather than ATP synthesis. Fd⁻⁻, reduced ferredoxin; Hyd, bifurcating hydrogenase (yellow spot); Ech, energy converting [NiFe] hydrogenase.
The formation of 1 ATP/glutamate would be possible via SLP without using bifurcation and Rnf. A route via 2-oxoglutarate and succinyl-CoA could lead to methylmalonyl-CoA that decarboxylates to propionyl-CoA: Glutamate $+ 2 \text{H}_2\text{O} + \text{H}^+ \rightarrow \text{NH}_4^+ + 2 \text{CO}_2 + \text{Propionate}^\cdot + \text{H}_2$; $\Delta G^\circ = -19 \text{ kJ/mol glutamate}$; $\Delta G^\circ (1 \text{ mM}) = -53 \text{ kJ/mol glutamate}$.

Thus SLP from propionyl-CoA via propionylphosphate generating 1 ATP would be possible only in a consortium with syntrophic bacteria and methanogenic archaea. In addition 1 $\Delta \mu_{\text{Na}^+}$ could be conserved by the sodium translocating methylmalonyl-CoA decarboxylase and used for the sodium-glutamate symporter, both of which are

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**Fig. 13.** Ethanol–acetate fermentation of *C. kluyveri*. A, at high ethanol and acetate concentrations; B, at low ethanol and acetate concentrations. At low substrate concentrations RnfA–G is not operative and the F$_{1}$F$_{0}$ ATPase functions in the direction of ATP hydrolysis rather than ATP synthesis. The organism contains a NAD$^+$ specific (black arrow) and a NADP$^+$ specific (orange arrow) β-hydroxybutyryl-CoA dehydrogenase whose activities are regulated. Fd, ferredoxin; B/E, BcdA–EtfBC complex (yellow spot); Nfn, NfnAB complex (orange spot).
present in *A. fermentans* [108,111]. However, the also required coenzyme B12-dependent methylmalonyl-CoA mutase could not be detected in the genome.

4.5. Methane formation from CO2 and H2 by methanogenic archaea

Methanogens growing on H2 and CO2 generally do this in environments where the H2 partial pressure is very low, near 10 Pa. Under these conditions the free energy change associated with CO2 reduction to methane is about $-40 \text{ kJ/mol}$, which is only sufficient to drive the phosphorylation of 0.5 ADP. Substrate level phosphorylation is not involved (Fig. 15) [29].

In the energy metabolism there are two coupling sites: (i) the exergonic transfer of the methyl-group of methyl-tetrahydromethanopterin to coenzyme M ($\Delta G^\text{o'} = -30 \text{ kJ/mol}$) catalyzed by the membrane associated MtrA–H complex and coupled with the build-up of an electrochemical sodium ion potential [112], and (ii) the coupled reduction of ferredoxin and of the CoM-S-S-CoB heterodisulfide with 2 H2 via electron bifurcation catalyzed by the MvhADG–HdrABC complex [55] (Fig. 5). The reduced ferredoxin is required for the reduction of CO2 to formyl-methanofuran, which is the first step in methanogenesis from CO2. The redox potential $E^\circ$ of the CO2/formyl-methanofuran couple is $-500 \text{ mV}$. An energy converting hydrogenase (Section 3.2) replenishes the reduced ferredoxin required for the anabolic reduction of CO2 to pyruvate which involves a ferredoxin-dependent reduction of CO2 to CO ($E^\circ = -520 \text{ mV}$) and a ferredoxin-dependent reduction of acetyl-CoA + CO2 to pyruvate ($E^\circ = -500 \text{ mV}$) [29,53].

The methanogens growing on H2 and CO2 using the pathway as outlined in Fig. 15 do not contain cytochromes. Methanogens with cytochromes cannot grow on H2 and CO2 at H2 partial pressures as low as 10 Pa [29,55]. They couple chemiosmotically the exergonic reduction of CoM-S-S-CoB with H2 and the endergonic reduction of ferredoxin with H2 [113].

4.6. Acetic acid formation from CO2 and H2 by acetogenic bacteria

The threshold concentration of acetogens for H2 (200 Pa) is much higher than that of methanogens (10 Pa) [50]. At 200 Pa the free energy change associated with acetogenesis from H2 and CO2 is only about $-35 \text{ kJ}$ which is sufficient for the synthesis of about 0.5 mol ATP. How these 0.5 mol ATP are generated is still not known, neither in *A. woodii* (contains no cytochromes) nor in *M. thermoacetica* (contains cytochromes), which are the best studied model organisms (Fig. 16). In both organisms the ATP generated in the acetate kinase reaction via SLP is required for the activation of formate to formyl-tetrahydrofolate (formyl-H4F). Thus no net ATP is formed via SLP.

It has recently been proposed, how in *A. woodii* energy is conserved [50]. *A. woodii* contains an electron bifurcating [FeFe]hydrogenase catalyzing the coupled reduction of NAD+ and ferredoxin with 2 H2, similar to the HydABC complex in Section 2.2. Two of the enzymes involved in CO2 reduction to acetic acid are NAD+-specific (methylene-H4Fe-dehydrogenase and methylene-H4F-reductase), one is ferredoxin specific (CO dehydrogenase) and one (formate dehydrogenase) is in a complex with a second [FeFe]hydrogenase. The organism contains an energy conserving RnfA–G complex [82] rather than an energy converting [NiFe]hydrogenase complex [50]. With this information the metabolic scheme shown in Fig. 16 can be drawn predicting that per mol acetic acid formed from H2 and CO2 only 0.25 mol ATP is generated. The scheme does not include the proposal that the methylene-H4F-reductase could be electron bifurcating [50] because it could not yet be demonstrated that the methylene-H4Fe-reductase couples the exergonic reduction of methylene-H4F ($E^\circ = -200 \text{ mV}$) by NADH with the reduction of ferredoxin.

*M. thermoacetica* also contains the electron bifurcating [FeFe]hydrogenase HydABC catalyzing the coupled reduction of NAD+ and ferredoxin with 2 H2 and, in addition, an electron bifurcating transhydrogenase catalyzing the coupled reduction of two NADP+.
with reduced ferredoxin and NADH [28] (Fig. 16). Two of the enzymes involved in CO₂ reduction to acetic acid are NADP⁺ specific (formate dehydrogenase and methylene-H₄F dehydrogenase), one enzyme is ferredoxin specific (CO dehydrogenase) and one is of unknown electron donor specificity (methylene-H₄F reductase). It has been proposed that the exergonic reduction of methylene-H₂F to methyl-H₄F (ΔE' = −200 mV) with H₂ could somehow drive the reduction of ferredoxin, which in turn could be oxidized by protons via the energy converting [NiFe]-hydrogenase complex (EchA-G) present in *M. thermoacetica* which lacks genes for an energy conserving RnfA–G complex [28]. Experimental evidence for this coupling mechanism is, however, not yet available.

### 5. Discussion

In this review we have described that many anaerobes contain cytoplasmic flavin-containing enzymes that catalyze the reduction of ferredoxin by an electron donor with a more positive redox potential than that of ferredoxin (E°_donor − E°_ferredoxin = ΔΔE > 0). This is accomplished by coupling the oxidation of the same donor by an acceptor with a more positive redox potential than that of the donor (E°_acceptor − E°_donor = ΔΔE > 0). This process, called flavin based electron bifurcation, is only possible if ΔΔE ≥ ΔΔE₁, otherwise it cannot proceed. The flux of the bifurcation should be proportional to the difference ΔΔE₂ − ΔΔE₁, which is equivalent to the overall ΔΔG° of the bifurcation. Via the oxidation of the reduced ferredoxin thus generated, either with NAD⁺ (energy conserving reduced ferredoxin: NAD⁺ oxidoreductase, Rnf, mainly in bacteria) or with H⁺ (energy converting [NiFe]hydrogenases in archaea and bacteria) the negative electron potential of the reduced ferredoxin is converted into an electrochemical proton or sodium ion potential which in turn drives the phosphorylation of ATP. The clostridial [FeFe]hydrogenases also contribute to energy conservation, because the reduction of protons to H₂ saves substrate as electron acceptor and thus increases substrate oxidation to ‘energy rich’ intermediates for SLP. We showed with 9 examples how via flavin-based electron bifurcation and/or chemiosmotic coupling the “last drop of free energy is squeezed out of the fermentations for the synthesis of as much ATP as possible” [47]. We did not mention until now that there may be exceptions from this rule.

*Clostridium propionicum* ferments 3 alanine to 3 ammonia, 1 acetate, 1 CO₂ and 2 propionate via the acrylyl-CoA pathway. A related pathway *Megasphaera elsdenii* converts 6 lactate to 4 CO₂, 2 H₂, 1 acetate, 1 propionate, 1 butyrate, 1 valerate and traces of caproate. The free energy changes associated with these fermentations are −49 kJ/mol alanine and −46 kJ/mol lactate. This is sufficient to allow for the phosphorylation of at least ½ ADP/alanine or lactate but all available evidence indicates that only about 0.5 ATP is generated via SLP and electron transport phosphorylation without bifurcation. The purified propionyl-CoA dehydrogenase/Etf complex was reported to catalyze the reduction of acrylyl-CoA with NADH without the requirement of ferredoxin [114], although its amino acid sequences share high identities to those of the Bcd–Etf complex from *C. kluyveri*. Similarly, the purified Etf from *M. elsdenii* in combination with the butyryl-CoA dehydrogenase appears to mediate the reduction of crotonyl-CoA by NADH without ferredoxin [115], though this system is closely related to the bifurcating system from *A. fermentans* (see Section 2.1). Both, *M. elsdenii* and *A. fermentans*, belong to the family *Acidaminococcaceae*, the only Gram-negative family among the phylum *Firmicutes*. Finally, the growth yield of *Clostridium homopropionicum* fermenting alanine via acrylyl-CoA to propionate is about half as high as that of *Propionibacterium freudenreichii* which uses the methylmalonyl-CoA pathway for propionate production [116]. Hence, the propionyl-CoA dehydrogenase/EtfBC complexes from *C. propionicum* and *C. homopropionicum* as well as EtfBC from *M. elsdenii* might be regarded as mutants that lost the ability of bifurcation to cope with the highly reactive electrophilic and therefore toxic acrylyl-CoA [117,118]. These examples strongly indicate that organisms avoid bifurcation with acrylyl-CoA, though its redox potential of ΔE° = +69 mV would favor this process even better than crotonyl-CoA (ΔE° = −10 mV) [10].
bifurcation the steady state concentration of the toxic acrylyl-CoA should be much lower. In this respect it is of interest that some facultative bacteria contain enzyme complexes with the cytochrome b$_6$f$_1$ module, which do not use quinone-based electron bifurcation [119], probably due to thermodynamic reasons (Section 2.6).

The four cytoplasmic enzyme complexes shown to catalyze electron bifurcation reactions have in common that they all contain at least one flavin, which is the reason why we assume that electron bifurcation in all four enzyme complexes is flavin based. But this does not necessarily have to be true. It has been suggested that in addition to ubiquinone and flavins other compounds such as WIV and MoIV and possibly even H$_2$ could be electron bifurcating [47,120,121]. The formate dehydrogenase of NAD$^+$ with formate, is most probably a molybdenum protein (Section 2.5) [61]. Although this enzyme has not yet been purified and characterized, we envisage that this enzyme could catalyze a molybdenum-based electron bifurcation reaction. The current hypotheses of the origin of life mainly focus on a chemolithoautotrophic start with the reduction of atmospheric CO$_2$ to acetate by hydrogen produced by reduction of H$_2$O with Fe$^{II}$ in the earth’s crust. As shown in Fig. 16, acetogenesis relies on electron bifurcation, due to the inability of hydrogen, especially at pressures far below 10$^5$ Pa, to efficiently reduce CO$_2$ to CO. Hence, bifurcation might have driven the evolution of the first biochemical pathways [121].

Finally we want to add B$_{12}$ to the list of possibly electron bifurcating compounds. B$_{12}$ with its two-redox transitions from cob(I)alamin via cob(II)alamin to cob(III)alamin could also do the electrochemical trick of oxidation triggered reductions [47]. Anaerobic bacteria growing on H$_2$ with halogenated compounds as electron acceptors involve cob(1)alamin in reductive dehalogenation reactions [112–125]. How this exergonic reaction is coupled with energy conservation is not yet known. B$_{12}$-based electron bifurcation in dehalorespiration is therefore something to think about.

**Fig. 16.** Acetate formation from H$_2$ and CO$_2$ by *Acetobacterium woodii* (does not contain cytochromes) and *Moorella thermoacetica* (contains cytochromes). HCO$_3^-$, formyl-tetrahydrofolate; CH$_2$G, energy converting hydrogenases; RnfA, -G. 

\[
\text{4H}_2 + 2\text{CO}_2 \rightarrow \text{CH}_3\text{COOH} + 2\text{H}_2\text{O} \quad \Delta G^\circ = -95 \text{kJ/mol} : \quad \Delta G^\circ = -35 \text{kJ/mol at pH}_7 = 200 \text{Pa}
\]

0.25 (?) ATP/Acetate

? ATP/Acetate

Note added in proof

In the meantime it was found that Bcd together with Etf from *M. elsenedi* catalyze the crotonyl-CoA-dependent reduction of ferredoxin with NADH only under strictly anaerobic conditions (N. Pal Chowdhury and W. Buckel).

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