Author for correspondence:
Eric S. Boyd
e-mail: eboyd@montana.edu

Bioenergetic constraints on the origin of autotrophic metabolism

Eric S. Boyd[1], Maximiliano J. Amenabar[1], Saroj Poudel[1] and Alexis S. Templeton[2]

[1]Department of Microbiology and Immunology, Montana State University, Bozeman, MT 59717, USA
[2]Department of Geological Sciences, University of Colorado, Boulder, CO 80309, USA

ESB, 0000-0003-4436-5856; AST, 0000-0002-9670-0647

Autotrophs form the base of all complex food webs and seemingly have done so since early in Earth history. Phylogenetic evidence suggests that early autotrophs were anaerobic, used CO_2 as both an oxidant and carbon source, were dependent on H_2 as an electron donor, and used iron-sulfur proteins (termed ferredoxins) as a primary electron carrier. However, the reduction potential of H_2 is not typically low enough to efficiently reduce ferredoxin. Instead, in modern strictly anaerobic and H_2-dependent autotrophs, ferredoxin reduction is accomplished using one of several recently evolved enzymatic mechanisms, including electron bifurcating and coupled ion translocating mechanisms. These observations raise the intriguing question of why anaerobic autotrophs adopted ferredoxins as central electron carriers only to have to evolve complex machinery to reduce them. Here, we report calculated reduction potentials for H_2 as a function of observed environmental H_2 concentration, pH and temperature. Results suggest that a combination of alkaline pH and high H_2 concentration yield H_2 reduction potentials low enough to efficiently reduce ferredoxins. Hyperalkaline, H_2 rich environments have existed in discrete locations throughout Earth history where ultramafic minerals are undergoing hydration through the process of serpentinization. These results suggest that serpentinizing systems, which would have been common on early Earth, naturally produced conditions conducive to the emergence of H_2-dependent autotrophic life. The primitive process of hydrogenotrophic

methanogenesis is used to examine potential changes in methanogenesis and Fd reduction pathways as these organisms diversified away from serpentinizing environments.

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1. Introduction

Ferredoxins (Fds) are ubiquitous in biology and link oxidation and reduction reactions in a diversity of metabolisms, including those of anaerobes, aerobes, phototrophs, chemotrophs, autotrophs and heterotrophs [1]. These small soluble proteins have active sites composed of iron–sulfur (Fe–S) clusters (i.e. 2Fe-2S or 4Fe-4S clusters) [2] that can accept or donate electrons with a concomitant change in the oxidation state of the iron atom(s) between $+2$ and $+3$. These proteins have been widely argued to be among the most primitive of electron carriers [3]. The electrochemical potential of Fe–S clusters, including those of Fds, can range from -0.60 to $+0.50$ V [4], thereby encompassing the array in potentials associated with oxidation–reduction reactions that fuel life in reduced environments reminiscent of those on early Earth as well as oxidized environments that characterize surface locales on modern Earth [5].

Genes encoding Fds are enriched in the genomes of anaerobes relative to aerobes and facultative anaerobes [6], a finding that is consistent with the central role of Fds in anaerobic energy metabolism [7]. This is particularly true for the genomes of strictly anaerobic, hydrogenotrophic methanogens [6], a group of organisms often advocated as having the most primitive of extant metabolisms [8,9]. Rationales for the primacy of hydrogenotrophic methanogens are manifold. Firstly, isotopic analysis of methane in fluid inclusions in ancient rocks dated to approximately 3.46 Ga suggest the presence of ancestors of methanogens at this time [10] and phylogenetic data suggest that these ancestral cells were likely dependent on H_2 as electron donor [9,11]. Secondly, hydrogenotrophic methanogens can use CO_2 as their sole source of carbon as well as their electron acceptor, resulting in the production of methane (CH_4). This potentially alleviated a major barrier for early autotrophic life since CO_2 was likely widely available on a volcanically active early Earth while organic carbon and other potential oxidants (e.g. oxygen, nitrate, sulfate) capable of supporting life are likely to have been far less available [12]. Finally, methanogens rely on the reductive acetyl CoA pathway, or the Wood Ljungdahl (WL) pathway [8], for carbon fixation. The WL pathway forms acetyl CoA from CO_2 in an overall thermodynamically favourable reaction [13] and has been suggested to be the most ancestral of extant carbon fixation pathways [8,9]. Thus, methanogens and other early evolving organisms that use the WL pathway (e.g. acetogens) do not expend the limited energy supplied by their primitive anaerobic metabolisms to generate reduced carbon like other autotrophs operating less efficient pathways.

The typical Fds involved in the energy metabolism of hydrogenotrophic methanogens and acetogens are highly reduced, having standard state (25°C, pH 7.0, 1 M reactant and product concentrations) reduction potentials ($E°$) relative to the standard hydrogen electrode of approximately -0.41 to -0.50 V [14,15]. This is possibly a consequence of selection to harbour an Fe–S cluster with a low enough potential to allow for the initial reduction of CO_2, which is estimated to be approximately -0.50 V [16]. However, the $E°'$ of H_2, the source of electrons supporting the energy metabolism of these cells and organic carbon synthesis via the WL pathway, is approximately -0.41 V [17,18], which is not of low enough potential to efficiently reduce the Fe–S cluster(s) in low potential Fd (Reaction 1).

$$0.5H_2 + Fd_{ox} \leftrightarrow H^+ + Fd_{red}^{1-} \qquad \text{(Reaction 1)}$$

This is particularly true considering that Fd in cells is generally 90% reduced [19], thus favouring preservation of reactants.

Nonetheless, numerous anaerobic, hydrogenotrophic methanogens and acetogens have been characterized in laboratory and natural settings (e.g. [17,20]). How do contemporary anaerobic cells reduce low potential Fd with H_2 as an electron carrier? At least two distinct enzymatic

mechanisms have evolved to overcome this thermodynamic barrier in H_2 oxidizing anaerobic autotrophs: electron bifurcating Fd reduction and ion (proton or sodium) translocating Fd reduction [7,14]. Electron bifurcation involves the endergonic reduction of low potential Fd by an electron donor with a higher potential. This is only possible when the same donor is also coupled to concomitant exergonic oxidation by an even higher potential acceptor [17,18]. Twelve multimeric bifurcating enzyme complexes have been described to date and all are involved in the reduction of Fd; however, only three of these multimeric complexes are known to use H_2 as the hydride donor [7,15,21]. These include the trimeric/tetrameric [FeFe]-hydrogenases, the hexameric [FeFe]-hydrogenase-CO_2 reductase and the hexameric [NiFe]-hydrogenase-heterodisulfide reductase (Mvh-Hdr). In ion translocating Fd reduction, the unfavourable thermodynamics associated with Fd reduction with H_2 are overcome by coupling the chemical reaction to depletion of an electrochemical ion (i.e. sodium ion or proton) gradient across a membrane [7]. Two classes of membrane-bound enzyme complexes are known to catalyse this function: the energy converting [NiFe]-hydrogenases (Ech or Eha/Ehb) and the Fd:NAD^+ oxido-reductase (Rnf). Both complexes couple the reversible reduction of Fd and oxidation of H_2 (Ech/ Eha/Ehb) or NADH (Rnf) with the translocation of sodium ions or protons into the cell [22–24].

Based on the fundamental role of electron bifurcating complexes and ion translocating Fd oxidoreductase enzymes in the energy metabolism of hydrogenotrophic methanogenic or acetogenic anaerobes [7], these enzymes or processes are often advocated as having been integrated into the energy metabolism of primitive lifeforms on early Earth [25–27]. In support of this hypothesis, the genomes of hydrogenotrophic acetogens and methanogens that could be classified based on previous physiological characterization encode one or more H_2-dependent electron bifurcating or ion translocating Fd oxidoreductase enzyme homologs (electronic supplementary material, table S1). However, a recent analysis of the taxonomic distribution and evolutionary history of bifurcating enzymes revealed that none of the twelve known bifurcating complexes are likely to have been a property of the Last Universal Common Ancestor (LUCA) of Archaea and Bacteria [28]. Rather, evidence suggests that the twelve known bifurcating complexes emerged independently multiple times after the divergence of Archaea and Bacteria from the LUCA. Likewise, phylogenetic analyses of Ech/Eha/Ehb suggest that these enzymes are likely to have emerged in methanogenic Archaea after their divergence from LUCA, with acquisition among Bacteria taking place more recently [11]. Finally, Rnf enzymes are found in several lineages of anaerobic Bacteria but are only found in several late diverging methanogenic Archaea (e.g. Methanosarcinales; [23]) and are absent in more ancestral methanogens (e.g. Methanococcales, Methanobacteriales; electronic supplementary material, table S1), suggesting that they too may be recently evolved. Collectively, these observations raise key new questions regarding how Fd was reduced in ancestral, autotrophic and anaerobic cells such as methanogens prior to the advent of complex mechanisms that allow for its reduction in contemporary cells.

Here, we provide thermodynamic calculations of the potential of Fd and H_2 across a range of environmental conditions to identify those that may be suitable for direct reduction of Fd with H_2 in early evolving anaerobic autotrophs in the absence of bifurcating or ion-translocating hydrogenases. Results are discussed at the level of the possible ancestral pathways that enabled autotrophy in early evolving hydrogenotrophic anaerobes and the role of ion-translocating and bifurcating hydrogenases in the diversification of ancestors of anaerobic, H_2-dependent autotrophs away from environmental realms that may have favoured the direct reduction of Fd with H_2.

2. Material and methods

(a) Relationship between the mode of carbon metabolism and distribution of bifurcating enzyme homologs

A database of organisms with complete genomes ($n = 4,588$) created in our previous analysis of the taxonomic distribution on bifurcating enzyme homologs [28] was subjected to classification

of their ability to integrate oxygen into their energy metabolism based on information acquired from the Department of Energy's Integrated Microbial Genomes and Microbiomes (DOE-IMG). All identified anaerobic taxa were further characterized as hydrogen oxidizing autotrophic acetogens or methanogens based on the available literature (electronic supplementary material, table S1). This curated list of organisms was then cross-referenced with our published database describing the distribution of bifurcating enzyme homologs in their genomes [28]. In addition, the genomes of these organisms were screened for homologs of ferredoxin:NAD$^+$ oxidoreductase (Rnf) and the group 4 membrane-bound [NiFe]-hydrogenase (Ech/Eha/Ehb). Briefly, Rnf protein homologs from *Azotobacter vinelandii* (ACO81179-ACO81185) and a homolog of the large subunit of group 4 [NiFe]-hydrogenase from *Pyrococcus abyssi* (WP_010867842) were subjected to analysis via phmmer available in the HMMER (v.3) software package (hmmer.org) to extract protein homologs. Extracted homologs of [NiFe]-hydrogenases were aligned using Clustal Omega [29] and filtered to contain the conserved residues that demarcate these enzymes as identified in a previous study [30].

(b) Redox potential calculations

The ability of a compound to donate or accept electrons is dictated by its redox potential (E), which can be calculated using equation (2.1):

$$E = E^\circ + 2.303 \left(\frac{RT}{nF} \right) \log \frac{\alpha_{\text{Ox}}}{\alpha_{\text{Red}}}, \tag{2.1}$$

where E° represents the standard redox potential of reaction in volts (V), R designates the ideal gas constant (approx. $8.314 \, \text{J} \, \text{mol}^{-1} \, \text{K}^{-1}$), T is the temperature in Kelvin (K), n is the number of electrons transferred in the reaction, F is the Faraday constant (approx. $96\,485 \, \text{J} \, \text{V}^{-1} \, \text{mol}^{-1}$), α_{Ox} is the activity of the oxidized form of the compound and α_{Red} is the activity of the reduced form. Redox potential calculations for H_2 were performed across a range of H_2 activities (0.0001 to 10 mM), temperatures (0 to 100°C) and pH values (4.0; 7.0; 10) using equation (2.1). In addition, the redox potential of H_2 in natural environments was calculated using reported (electronic supplementary material, table S2) dissolved H_2 concentrations, temperatures and pH values. Activity coefficients were assumed equal to one for all dissolved compounds (activity = concentration) since the ionic strength was not always reported.

3. Results and discussion

The non-standard state electrochemical potential (E) of a half-reaction is dependent on how the E° is modified by temperature, pressure, and the concentrations of reactants and products. In the case of the half-reaction describing the oxidation of H_2 (Reaction 2), the potential is dependent on pH since protons (H^+) are a product of the specified reaction. Thus, the potential of H_2 (thermodynamic drive toward products) will be lower in environments with lower H^+ activity.

$$H_2 \rightarrow 2e^- + 2H^+. \tag{Reaction 2}$$

Using thermodynamic data, we calculated the E of the half-reaction describing the oxidation of H_2 (Reaction 2, hereafter referred to as the potential of H_2) as a function of temperature, proton concentration (i.e. pH), and H_2 concentration to identify conditions that may be conducive to the direct reduction of low potential Fds associated with anaerobic autotrophs ($E^\circ = -0.41$ to -0.50 V at pH 7.0, 25°C), both today and in the geological past. To facilitate comparison of Fd reduction potentials relative to Reaction 1, we also calculated the E of Fds across a range of temperatures and pH (figure 1). The temperature dependence of the E of Fds ($E^\circ = -0.41$ to -0.50 V) was calculated by extrapolating measurements of Fd purified from *Pyrococcus furiosus* as a function of temperature (-0.00123 V/°C) [31]. The pH dependence of the E of Fds was calculated by extrapolating the maximum measured effect of pH on the measured E of Fd (0.030 V/pH unit) [32] purified from a variety of taxa (range = 0.002 to 0.030 V/pH unit) [32–34]. These calculations
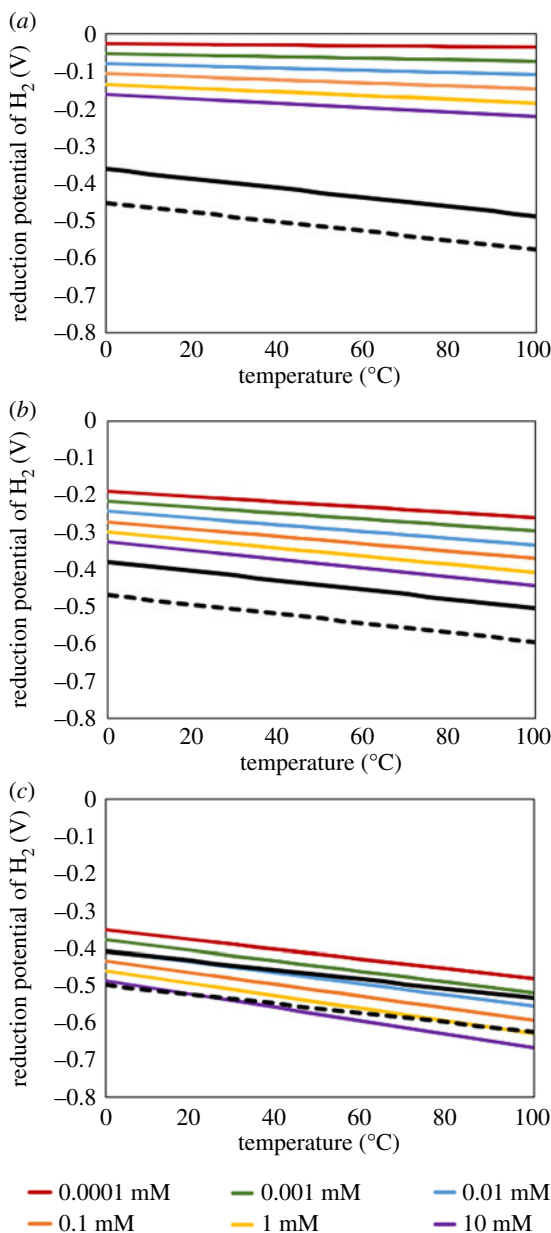
Figure 1. Reduction potential of H_2 (Reaction 1) as a function of temperature and H_2 concentration in water with a pH of 4.0 (*a*), 7.0 (*b*) and 10.0 (*c*). The black line in each panel depicts the calculated midpoint potential of Fds with an $E^{\circ\prime}$ of -0.41 V while the dashed black line in each panel depicts the calculated midpoint potential of Fds with an $E^{\circ\prime}$ of -0.50 V. Calculations of the midpoint potential of Fds assumed a concentration of 1 M. The temperature and pH dependence of the midpoint potentials calculated for Fds were assumed to be 0.00123 V/°C [31] and 0.030 V/ pH unit [32], respectively. (Online version in colour.)

assumed a single electron transfer per Fd (Reaction 1) since ancestral Fds are thought to have comprised a single Fe–S cluster [3] and assumed a constant concentration of Fd (1 M). Importantly, ionic strength has also been shown to influence the E of purified Fd [34]. However, the inverse relationship between ionic strengths ranging from 0 (slightly lower than freshwater) to 1.0 (greater than seawater) and the measured E of Fd is minimal (0.061 V) and is therefore not considered further in the calculations presented here.

The calculated E of H_2 dissolved in water with a pH of 4.0, when considered over a range of dissolved H_2 concentrations from 100 nM to 10 mM and temperatures from 0 to 100°C, ranged from -0.03 V to -0.22 V (figure 1*a*). More negative potentials were associated with higher concentrations of dissolved H_2 and higher temperature; the effects of these parameters were additive. In water with a pH of 7.0 and over the same range of dissolved H_2 concentrations and temperatures, the E of H_2 varied from -0.19 V to -0.44 V with more negative potentials in solutions with higher concentrations of dissolved H_2 and higher temperature (figure 1*b*). Finally, dissolved H_2 in water with a pH of 10.0 and over the same range of dissolved H_2 concentrations and temperatures had calculated E that ranged from -0.35 V to -0.67 V (figure 1*c*). In water with a pH of 10.0, the calculated E of H_2 at a concentration of greater than 1 mM would be low enough to efficiently reduce low potential Fd ($E° = -0.41$ V) at temperatures greater than 25°C. The reduction of lower potential Fd ($E° = -0.50$ V) at pH 10.0 requires higher H_2 concentrations (greater than 10 mM) and temperatures (greater than 100°C). As previously mentioned, the relationship between the E of Fd and pH was calculated using the maximum measured inverse slope of 0.030 V/pH unit. Thus, it is possible that efficient reduction of low potential Fds could take place in a setting with a lower pH, temperature, and H_2 concentration than those specified above. Alternatively, it is possible that the potential of Fds vary with pH in a more Nernstian-like manner, whereby a decrease in the potential of 0.059 V/pH unit would be expected. If so, a higher concentration of H_2, a higher pH, and/or temperature would be required to reduce low potential Fds than those specified above.

To identify environmental types with conditions that may support efficient reduction of low potential Fds, the dissolved H_2 concentrations (range = 2.3 nM–2.9 mM), temperatures (range = 0.4–90.0°C), and pH values (range = 2.9–12.4) of waters from various early Earth analogue environments that had temperatures less than 122°C, the current upper temperature limit for life [35], were compiled (electronic supplementary material, table 2). These environments were chosen because of (i) available temperature, pH and H_2 concentration data, (ii) their potential to host non-photosynthetic, chemosynthetic cells thought to reflect the earliest forms of life [9,11] and (iii) previous suggestions of their relevance as habitats supporting descendants of early forms of life [36–40]. The following environment types met these criteria: marine and terrestrial hydrothermal springs, subglacial environments overlying basalt bedrock, and marine and terrestrial subsurface environments where water is in contact with ultramafic rocks (i.e. serpentinites).

The calculated E of H_2, when plotted as function of temperature (figure 2*a*), pH (figure 2*b*) or H_2 concentration (figure 2*c*), from a variety of environments identified natural conditions that could conceivably favour the efficient direct reduction of low potential Fd with H_2. These include subsurface fluids in contact with ultramafic rocks in the Coast Range Ophiolite Microbial Observatory (CROMO) in the USA (green triangles), the Samail Ophiolite in the Sultanate of Oman (orange dots), and the Lost City hydrothermal field in the Atlantic Ocean (blue diamonds). Common to each of these three environmental types is a combination of alkaline pH (figure 2*b*) and high H_2 concentration (figure 2*c*) that span a range of temperatures (figure 2*a*). The alkaline pH and elevated H_2 concentrations in each of these environment types is due to hydration of ultramafic rock rich in olivine and pyroxene, which stimulates a series of reactions collectively known as serpentinization that generate hydroxide ions and molecular H_2 [41]. The process of serpentinization is thought to have been more prevalent on early Earth where highly reduced mineralogy was likely more widespread in an undifferentiated crust [36,42]. In turn, and as shown here, hyperalkaline, H_2-rich conditions are potentially conducive to the direct and efficient reduction of Fd in anaerobic autotrophs if suitable catalysts are available, leading to the suggestion that serpentinizing systems hosted the earliest evolving, Fd- and H_2-dependent anaerobic autotrophs.

The primary catalysts or enzymes involved in the reversible oxidation of H_2 in modern cells are [FeFe]- and [NiFe]-hydrogenases. While [FeFe]-hydrogenases are unlikely to be a property of the LUCA [43], [NiFe]-hydrogenase have been suggested to have been associated with the LUCA where they likely functioned in H_2 oxidation [9,11]. It is worth noting that both Fe and
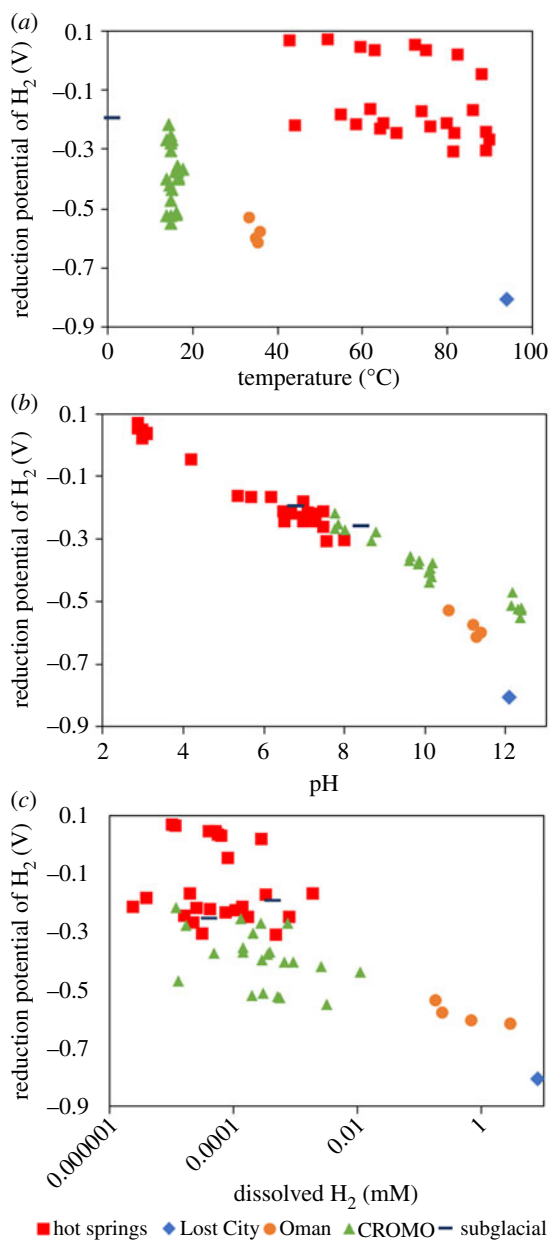
Figure 2. Reduction potential of H_2 (Reaction 1) in early Earth analogue natural environmental samples as a function of temperature (*a*), pH (*b*) and H_2 concentration (*c*). H_2 concentrations, pH, temperatures and calculated *E* for H_2 are presented in electronic supplementary material, table S2. (Online version in colour.)

Ni, the metals that form the active site of [NiFe]-hydrogenase, are common components of serpentinite rocks [36]. H_2 oxidation via [NiFe]-hydrogenases in contemporary hydrogenotrophic methanogens occurs in the cytoplasm (figure 3) [17], and this may have also been true for ancestors of these cells. If primordial hydrogenases were also localized in the cytoplasm, the calculations presented above must be modified to accommodate shifts in the reduction potential of H_2 when it is oxidized within the cell. This is because contemporary cells tend to maintain or buffer the pH of the cytoplasm within a narrower range than the pH outside the cell [44] but cannot regulate temperature or H_2 concentration. The same is likely to be true for primitive autotrophic cells.
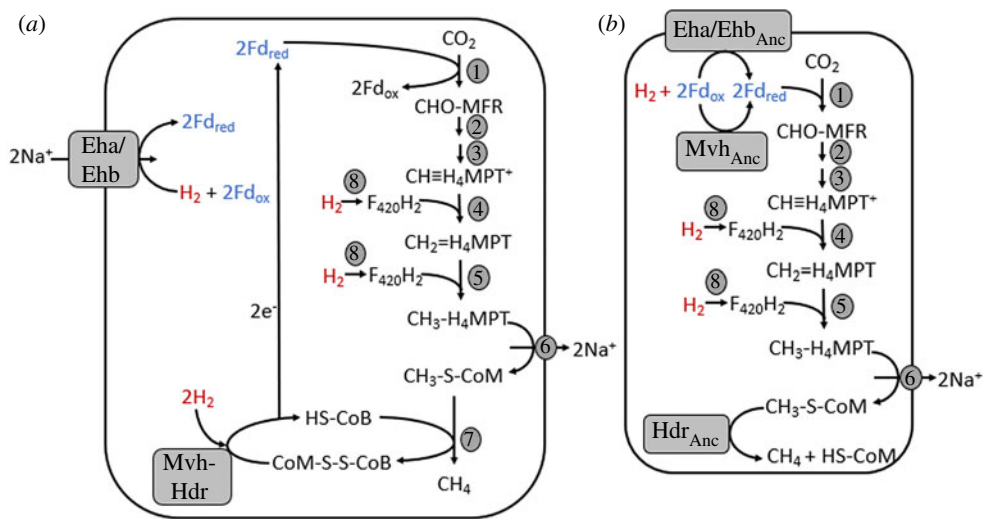
Figure 3. A simplified depiction (*a*) of the energy metabolism of contemporary hydrogenotrophic methanogens as modified from Thauer *et al.* [17] and the linear pathway (*b*) as proposed herein for the ancestor of hydrogenotrophic methanogens. The bifurcating Mvh-Hdr and the ion translocating Eha/Ehb[NiFe]-hydrogenases are depicted, as is the proposed non-bifurcating ancestor of Mvh (Mvh$_{Anc}$). Enzymes and their enzyme category numbers (EC) shown as numbers are as follows: 1, formylmethanofuran (MFR) dehydrogenase (EC: 1.2.7.12); 2, formylmethanofuran:tetrahydromethanopterin (H$_4$MPT) formyltransferase (EC: 2.3.1.101); 3, Methenyltetrahydromethanopterin cyclohydrolase (EC: 3.5.4.27); 4, F$_{420}$H$_2$-dependent methylenetetrahydromethanopterin dehydrogenase (EC: 1.5.98.1); 5, coenzyme F$_{420}$-dependent N5,N10 methylenetetrahydromethanopterin reductase (EC: 1.5.98.2); 6, methyl-H$_4$MPT–coenzyme M methyltransferase (EC 2.1.1.86); 7, methyl coenzyme M reductase (EC 2.8.4.1); 8, coenzyme F$_{420}$ reducing [NiFe]-hydrogenase (EC 1.12.98.1). Enzyme abbreviations are as follows: Eha/Ehb$_{Anc}$, the putative ancestor of energy converting [NiFe]-hydrogenase (Eha/Ehb); Mvh$_{Anc}$, the ancestor of soluble [NiFe]-hydrogenase (Mvh); Hdr$_{Anc}$, the ancestor of soluble heterodisulfide reductase (Hdr). Fds are assumed to accept a single electron in the schematics. (Online version in colour.)

Hyperalkaliphiles maintain a cytoplasmic pH that is up to 2 units lower than that of their external environment [44], which would increase the E of H$_2$ as it diffuses into the cell. As an example, for a cell inhabiting a pH 12.0 environment (assumed cytoplasmic pH of 10.0) at 25°C to efficiently reduce low potential Fds with $E°$ of -0.41 V ($E = -0.50$ V) and -0.50 V ($E = -0.59$ V), H$_2$ would need to be at a concentration of 1 mM and 1 M, respectively. Again, these are likely maximum H$_2$ concentrations required based on the assumed relationship between E and pH (0.030 V/pH unit). Environments enriched in ultramafic minerals and undergoing active low-temperature serpentinization generate water with strongly alkaline pH, concentrations of H$_2$ that approach 10 mM, and are often at temperatures that exceed 25°C (i.e. Oman, Lost City; figure 2). However, based on available data, it is unclear whether a cytoplasmic pH of 10.0 is physiologically feasible in an autotroph since available CO$_2$ would speciate to carbonate (CO$_3^{2-}$) which has a low solubility and is not the carbon source for methanogenesis [17]. That said, methanogens have been grown with calcium or magnesium carbonate as their sole carbon source [45] in growth medium with a pH as high as 10.0 [46]. These organisms grow presumably by consuming CO$_2$ that had equilibrated in solution [45]. Moreover, putatively hydrogenotrophic methanogens have been detected in several environments undergoing active serpentinization [47], including well waters sampled from the Samail Ophiolite in Oman with pH of up to 11.3 [48,49]. This indicates that the low solubility of inorganic carbon in hyperalkaline environments and constraints that it imposes on the availability of CO$_2$ can be overcome, at least in modern methanogen cells.

Primitive autotrophic, H$_2$-dependent cells also faced the need to innovate mechanisms to shuttle electrons that minimized energy loss and that had potentials close to the H$_2$/H$^+$ redox

9

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couple. Low potential Fds would efficiently meet this need in environments where the E of H_2 was more reduced, such as in alkaline hydrothermal vents or other environments with ultramafic rocks that are undergoing active serpentinization. As a modern example, the calculated E of H_2 in fluids from hydrothermal vents at Lost City (pH 12.1, $[H_2] = 7.8$ mM, 94°C; electronic supplementary material, table S2) is -0.73 V (figure 2) or -0.66 V at a cytoplasmic pH of 10.0. These values are close to the calculated E of a typical anaerobic Fd, which is estimated to range from -0.58 to -0.67 V at 95°C at a cytoplasmic pH of 10.0. Importantly, it is also likely that ancestral Fds comprised a far simpler protein environment than contemporary Fds [3], perhaps consisting of as few as 7 amino acids [50,51]. Fe–S clusters in such primitive Fds would presumably be more solvent exposed, which in turn would be expected to poise their E more positive than contemporary anaerobic Fds [4]. This, in turn, may have allowed early autotrophic anaerobes to exploit H_2 as an electron donor in a broader range of environments where reduction of these primitive Fds was favourable, including those with lower H_2 concentrations or with slightly less alkaline pH where inorganic carbon would have been more bioavailable. However, as depicted in figures 1 and 2, environments with temperature, pH, and H_2 conditions that may have been favourable for the reduction of low potential Fds represent only a fraction of those that were likely available on early Earth. We suggest that the emergence of complex enzymes enabling electron bifurcating Fd reduction and ion (proton or sodium) translocating Fd reduction [7,14] was triggered by the diversification of anaerobic life away from environments that favoured efficient and direct Fd reduction and into environments where the thermodynamics and kinetics of Fd reduction with H_2 were less favourable. These would have included environments with lower H_2 concentrations, lower temperatures, less alkaline pH or a combination of these factors.

The emergence of electron bifurcating and ion-translocating [NiFe]-hydrogenase enzymes was accomplished by recruitment of flavoprotein/heterodisulfide reductase [28] and ion transporter modules [11], respectively, thereby providing a mechanism to reduce Fd and meet the bioenergetic needs of early anaerobic autotrophs in environments less favourable for direct and efficient enzymatic reduction of Fd with H_2. A likely consequence of these diversifications is the presence of electron bifurcating and/or ion-translocating [NiFe]-hydrogenase enzyme homologs involved in reversible H_2 oxidation in the genomes of all anaerobic H_2 oxidizing methanogens and acetogens (electronic supplementary material, table S1), suggesting that such mechanisms are obligate in these derived cells. It remains to be determined if anaerobic H_2-dependent methanogens or acetogens that lack genes coding for bifurcating and ion-translocating enzymes can be found in contemporary environments undergoing active serpentinization (i.e. Lost City or the Samail Ophiolite, Oman), or if approximately 3.8 Ga of evolution has erased all vestiges of these ancestral organisms. Further cultivation efforts focused on such organisms from environments with high pH and high H_2 concentrations are necessary to address this question directly. Alternatively, it may be possible to evaluate this question by reconstructing the metabolisms of difficult to cultivate methanogens or acetogens through assembly and binning of metagenomic sequence data followed by robust informatics and evolutionary analyses.

What might such an ancestral anaerobic H_2 oxidizing methanogen or acetogen cell comprise? If the hypothesis that bifurcating and ion-translocating [NiFe]-hydrogenase enzymes were not components of the progenitors of anaerobic autotropic cells [28] turns out to be correct and that these early autotrophic cells harboured metabolisms with characteristics that are reminiscent of contemporary H_2-dependent methanogens [9], a very different picture of the metabolism of primitive hydrogenotrophic methanogens emerges. Whereas the pathway of contemporary hydrogenotrophic methanogenesis is cyclic with the first and last steps linked by the oxidation and reduction of low potential Fd, respectively (figure 3a) [17], it is possible that an earlier version of this pathway was not cyclic but rather linear where the first and last steps were unlinked. For example, in the last step of hydrogenotrophic methanogenesis, soluble bifurcating Mvh-Hdr functions to couple the oxidation of H_2 with the simultaneous endergonic reduction of Fd and heterodisulfide in coenzyme M-coenzyme B (CoM-CoB); the reduced Fd that is generated is then used to drive the exergonic reduction of CO_2

and methanofuran to form N-formylmethanofuran [17]. Membrane-associated ion-translocating [NiFe]-hydrogenase enzymes (Ech/Eha/Ehb) function to supply additional reduced Fd for the initial reduction of CO_2 to form N-formylmethanofuran [17]. In an early Earth environment that favoured the direct reduction of Fd with H_2, a soluble [NiFe]-hydrogenase without bifurcating functionality (termed Mvh_{Anc}) could conceivably generate reduced Fd without coupling it to the reduction of CoM-CoB; CoM-CoB reduction could have been catalysed by a soluble heterodisulfide reductase (Hdr_{Anc}) (figure 3b). A membrane bound [NiFe]-hydrogenase without ion-translocating capabilities (termed Eha/Ehb_{Anc}) could have conceivably also supplied additional reduced Fd via direct reduction with H_2 (figure 3b), although it is unclear if such an enzyme would provide a selective advantage to a cell where Mvh_{Anc} was also present. As such, this putative ancestral methanogenesis pathway would be linear rather than cyclic. Moreover, the eventual emergence of bifurcating enzymes would help to explain the evolution of the cyclic pathway of methanogenesis that we see perpetuated in extant hydrogenotrophic methanogens from a simpler linear pathway in a manner similar to that proposed for the Kreb's cycle [52].

An origin for autotrophic life in an anoxic, alkaline environment undergoing active serpentinization, as suggested by the data presented here, is consistent with other data suggesting these environment types may have been conducive to the origin of autotrophic cells. For such cells to originate, they would have needed a constant source of chemically transducible energy, a requirement that is met in the form of abundant H_2 in environments undergoing active serpentinization [42] and/or in the form of naturally occurring proton gradients when serpentinized fluids (pH $\sim$ 10–11) mix with ocean waters in equilibrium with atmospheric CO_2 (pH $\sim$ 6) [37]. Likewise, electrons of sufficiently reduced potential would have needed to be efficiently captured for use in the reduction of CO_2 [15,17], a problem that is potentially overcome by H_2 oxidizing, Fd-reducing [NiFe]-hydrogenases and the low potentials associated with H_2 in hyperalkaline serpentinizing systems (figure 2). However, the hyperalkaline pH also presents a potential problem for primitive autotrophic cells since CO_2 speciates towards carbonate which is less bioavailable. Contemporary hydrogenotrophic methanogens, however, are capable of growth at pH of up to 10.0 [45,46] and have been detected in serpentinizing environments with even more alkaline pH (11.3) [48,49]; the same may have been true for their ancestors. It is possible that in such modern environments, autotrophic cells overcome limitations of inorganic carbon by using more soluble and hydrogenated forms of carbon such as carbon monoxide or formate, which can also be produced abiotically from H_2 and CO_2/HCO_3^- under serpentinizing conditions [53]. Indeed, formate has been detected in natural serpentinizing environments such as Lost City [54] and in Oman [48]. Under formatotrophic methanogenic growth conditions, oxidation of low potential H_2 may simply augment the metabolism of these autotrophic cells. Collectively, the characteristics of environments undergoing active serpentinization make them attractive targets for understanding the possible origin of autotrophic metabolism, and perhaps metabolism in general. The ideas presented here are testable by probing extant life inhabiting alkaline serpentinite hosted, H_2 rich environments via biochemical, genetic and physiological approaches and through metabolic reconstructions enabled by phylogenomic approaches.

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