Biochimica et Biophysica Acta 1827 (2013) 79–93

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Review

On the universal core of bioenergetics ☆

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ARTICLE INFO

Article history:
Received 29 June 2012
Received in revised form 6 September 2012
Accepted 7 September 2012
Available online 14 September 2012

Keywords:
Chemiosmosis
2nd law of thermodynamics
Electron transfer
Evolution
Quinone
Origin of life

ABSTRACT

Living cells are able to harvest energy by coupling exergonic electron transfer between reducing and oxidising substrates to the generation of chemiosmotic potential. Whereas a wide variety of redox substrates is exploited by prokaryotes resulting in very diverse layouts of electron transfer chains, the ensemble of molecular architectures of enzymes and redox cofactors employed to construct these systems is stunningly small and uniform. An overview of prominent types of electron transfer chains and of their characteristic electrochemical parameters is presented. We propose that basic thermodynamic considerations are able to rationalise the global molecular make-up and functioning of these chemiosmotic systems. Arguments from palaeogeochemistry and molecular phylogeny are employed to discuss the evolutionary history leading from putative energy metabolisms in early life to the chemiosmotic diversity of extant organisms. Following the Occam's razor principle, we only considered for this purpose origin of life scenarios which are contiguous with extant life. This article is part of a Special Issue entitled: The evolutionary aspects of bioenergetic systems.

1. Introduction

Life constantly regenerates highly structured cellular entities from almost randomly distributed chemical elements and small molecules by carrying out intricately organised chemical reactions. In thermodynamic terms, building structure and order is lowering of entropy. The fundamental laws of thermodynamics, however, stipulate that entropy in the universe is bound to increase, order is doomed to transform into randomness [1]. So does life violate the principles of thermodynamics? Of course it doesn't and we can all experimentally test this fact by depriving a live culture of bacteria of all sources of energy: rather than building up new biomass it will over time disintegrate due to the randomising power of temperature and light. Order does in fact arise from chaos even in inanimate, purely physical systems as we can witness, for example, in the emergence of the low entropy states of a tornado wind system out of calm and random atmospheric conditions. The prerequisites for entropy decreases are described by the second law of thermodynamics which states that $dG/dt = dH/dt - TdS/dt$.

dG/dt is always negative or at best zero (with G, H and S representing free energy, enthalpy and entropy of a closed system). Entropy decrease, that is, $dS/dt<0$, yields a positive -TdS/dt term which needs to be counterbalanced by an equal or higher negative dH/dt term. That means that build-up of order can only happen if a sufficiently high enthalpy was present in the system and is dissipated in the ordering process. For the tornado case, this enthalpy comes from the temperature gradient generated by unequal incidence of solar radiation.

Similarly, the *condicio-sine-qua-non* for life's ability to generate order from randomness is the availability of a sufficiently large reservoir of energy (*i.e.* the enthalpy term in the second law of thermodynamics; for a more detailed discussion emphasising the statistical interpretation of the 2nd law, see the article by Branscomb and Russell in this issue [1]). In all cellular life on our planet this energy comes in two kinds, (a) chemical disequilibrium between ATP and ADP and (b) electrochemical disequilibrium between strong metabolic reductants (mostly NAD(P)H and F_{420}) and the more oxidising environment. Both kinds of disequilibria are indispensable for life to exist and since they are constantly dissipated to maintain a high level of organisation, environmental sources of energy need to be tapped into to maintain the "intracellular batteries" charged, *i.e.* far from equilibrium. The mechanism converting environmental energy into intracellular disequilibria is bioenergetics.

Boringly uniform as the intracellular energy vectors may appear, the multitude of ways life has come up with to exploit a plethora of different environmental sources of energy is stunning and far from fully understood, both with respect to variability and molecular details of the

☆ This article is part of a Special Issue entitled: The evolutionary aspects of bioenergetic systems.

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http://dx.doi.org/10.1016/j.bbabio.2012.09.005

individual systems. In this article, we try and emphasise the commonalities of the diverse kinds of energy converting mechanisms which have become increasingly obvious during the last two decades. Against the background of the common features of all energy converting processes, the core of bioenergetics emerges as obeying simple and universal thermodynamic rules and employing universal molecular modules to capture free energy available in the environment.

We consider that these universal thermodynamic rules and universal molecular modules (or cofactors thereof) have been inherited from life's penultimate origins. Based on this premise, inferences on the likelihood of certain origin of life scenarios are proposed. This approach necessarily ignores scenarios which stipulate fundamental evolutionary metamorphoses distinguishing nascent life from its present form. The inherent inaccessibility to falsification of such non-contiguous models to our minds disqualifies them as scientific approaches as long as all attempts to relate extant life to primordial geochemistry in an explicit and experimentally testable way have not yet failed.

2. Chemiosmosis; life's way to make ATP

A few decades ago, substrate-level phosphorylation in traditional fermentative pathways was considered life's most ancestral strategy to keep ATP levels high. This notion was likely psychologically bolstered by the fact that substrate-level phosphorylation has a weak efficiency (inefficient things certainly are primitive, *i.e.* ancient, aren't they?) as compared to the sophisticated chemiosmotic systems mainly studied early on, that is, mitochondrial respiration and plant photosynthesis. The ingenious coupling of local chemical reactions through a delocalised, cumulative physicochemical entity, the proton-motive force, on the other hand seemed "highly evolved".

During recent years, those tables have turned as discussed in detail in [2]. Substrate-level phosphorylation was recognised as rather rare and the involved enzymes show no signs of deep ancestry. By contrast, chemiosmotic coupling was found ubiquitously in all cellular life and the enzyme performing ATP synthesis, the rotor-stator-type ATP synthase, is universal. Phylogenies of ATP synthases strongly support the presence of this enzyme in the Last Universal Common Ancestor (LUCA) of Bacteria and Archaea [3,4]. It is noteworthy that the chemiosmotic potential may also be harvested by a different, structurally much simpler enzyme, the so-called H^+-translocating pyrophosphatase [5–7], which does not synthesise ATP from ADP and inorganic phosphate (P_i) but PP_i from two molecules of P_i. Due to the small size of this latter enzyme and the resulting paucity of evolutionarily informative sites, phylogenies are (and may well remain) less robust than for the case of the rotor–stator system [8–10] and therefore presently prevent a definitive conclusion on the ancestry of this system.

The chemiosmotic potential in the vast majority of prokaryotes corresponds to a concentration gradient of protons (and in a few cases of sodium ions) over the cytoplasmic membrane. Since protons (and sodium ions) are positively charged, the chemiosmotic potential has an electrostatic and an osmotic component, the sum of which is referred to as the proton- (or sodium-) motive force and which amounts, in healthily growing cells to about 150 mV. Since ATP synthesis continuously collapses the proton gradient, chemiosmotic potential needs to be generated commensurate with its consumption.

Until a few years ago, the cytoplasmic membrane was considered the exclusive lipid barrier over which a chemiosmotic potential is generated in prokaryotes. The recent discovery that a subgroup of planctomycetes contains intracellular vesicles, organelle-like structures, dedicated to carry out anaerobic ammonia oxidation (ANAMMOX) [11–13], likely provides a striking exception to this general rule. Anammoxosome membranes appear to be energised and to contain ATP synthase. They thus represent a second chemiosmotic membrane system in these bacteria.

In the overwhelming majority of cases, build-up of chemiosmotic potential is achieved by the action of electron transport chains which are fuelled by electrochemical disequilibria between reducing and oxidising substrates present in the extracellular environment. This general scheme is depicted in Fig. 1A. (Bacterio)chlorophyll-based photosynthesis straightforwardly fits into this scheme if "present in the extracellular environment" is amended by "or generated by light". In fact, photosynthetic reaction centres provide reduced forms of quinones and ferredoxins as well as oxidised states of soluble electron carriers (cytochromes, cupredoxins, HiPIPs) which are components of the majority of non-photosynthetic chemiosmotic chains. This suggests that (Bacterio)chlorophyll-based photosynthesis emerged from a chemotrophic context. Can the scheme of Fig. 1A therefore be regarded as representing the general strategy of life for generating chemiosmotic potential? Quite but not completely since a second type of light-driven build-up of proton gradient relying on the membrane-integral protein Bacteriorhodopsin (BR) manages completely without electron transfer (Fig. 1B). In BR, a light-induced bond isomerisation triggers a gating mechanism which translocates protons across the membrane. This mechanism was first discovered and thoroughly studied in Haloarchaea [14–18]. Genomic surveys as well as *in situ* sequencing, however, have provided evidence that BR-harbouring prokaryotes are both phylogenetically diverse and ecologically widespread [19–22]. BR-mediated use of light energy to generate chemiosmotic potential may thus play a more important role than previously assumed, possibly promoted by the low metabolic cost of synthesising a single protein as well as the corresponding ease of laterally transferring BR-based photosynthesis [23]. Nevertheless, just as is true for (Bacterio)chlorophyll-based photosynthesis, this kind of energy conversion is necessarily limited to the photic zone, that is, a restricted area of our planet (not mentioning the temporal availability imposed by the diurnal cycle) as compared to the vast diversity of ecological niches colonised by life. BR (Fig. 1B) therefore may be regarded as the proverbial exception that proves the rule that life's truly universal way to harvest energy is by collapsing electrochemical disequilibria *via* proton-translocating electron transfer chains (Fig. 1A).

3. The redox span and electrochemical regime of a substrate couple directs the makeup of bioenergetic chains

3.1. The diversity of electrochemical regimes explored by bioenergetic chains

Obviously, not all couples of redox substrates yield the same amount of energy. The ΔG which can be drawn from a given couple depends both on the difference in standard electrochemical potential (ΔE_m) between the donor and acceptor and on the absolute concentration as well as the reduction/oxidation level of these substrates in a given environment (see formulae of Table 1). Since most of the detailed environmental parameters are ill-defined and moreover substantially variable over short periods of time, we will use ΔE_m as a first-approximation value to indicate whether certain substrate couples are poor or potent providers of energy.

In Fig. 2, we summarise basic electrochemical parameters of the molecules involved in a variety of bioenergetic mechanisms, such as standard redox midpoint potentials (at pH 7) of the reducing and oxidising substrates, the type and E_m, of the liposoluble hydrogen carriers (*i.e.* low potential menaquinones, high potential ubi-, plasto- or caldariellaquinones and the very low potential methanophenazines) and of the major metabolic reductants (such as NAD(P)H and $F_{420}H_2$). A number of prokaryotes contain multiple kinds of quinones and all quinones present are indicated for the cases where the exact quinone-type operating in a specific pathway isn't known.

Standard redox midpoint potentials at pH7 are of course only first approximations of the free energy available in a given electrochemical disequilibrium between donor and acceptor substrates [24]. Mass-

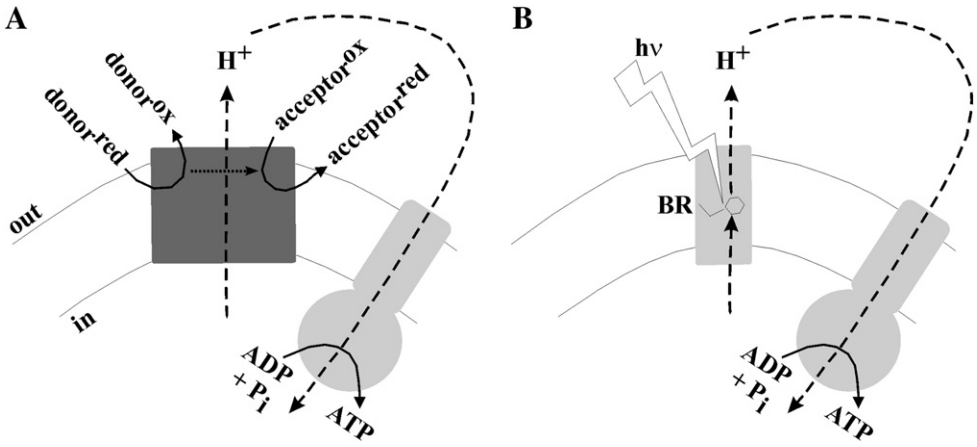

Fig. 1. Schematic representation of chemiosmotic systems performing ATP synthesis driven by a proton- (or Na^+) motive-gradient *via* the enzyme ATP synthase, based in (A) on electron transfer chains collapsing electrochemical disequilibria between reducing donor- and oxidising acceptor-substrates and, while doing so, translocating H^+/Na^+ ions across the chemiosmotic membrane. In (B), no electron transfer is involved and the ion gradient is generated *via* light energy through the retinal-containing rhodopsin protein.

action ratios, pH-values, number of transferred electrons all play a role in determining the true electrochemical ΔG (see Table 1) exploitable by an organism at a given time and in its specific growth environment. For example, in the third column from the left of Fig. 2, *i.e.* that deals with methanogenesis based on methanophenazine, we schematically indicate by a box elongated towards more negative potentials the larger redox drop from H_2 to CO_2 arising from higher partial pressures of H_2 (for a detailed discussion see [25]) by a box elongated towards more negative potentials with respect to that of the second column from the left. We are furthermore aware that the set of bioenergetic substrate couples depicted in Fig. 2 is far from exhaustive. Several of the bioenergetic mechanisms addressed in Fig. 2 weren't even known some 20 years ago, indicating the likelihood that many more such systems will be discovered in the future. However, the set of chosen bioenergetic chains and their representation *via* the E_m values of their donor/acceptor couples at pH7 visualises the different ranges of redox regimes covered by chemiosmotic systems and the strongly variable free energy available to different chains.

3.2. Ways to overcome endergonic barriers

Upward arrows in Fig. 2 indicate strongly endergonic and thus energetically challenging electron transfer steps. Chemiosmotic systems have come up with a number of tricks to overcome these endergonic barriers. Until recently it was considered that in all cases a part of the energy contained in an energised membrane is sidetracked to power these uphill redox reactions. However, some 40 years ago, Peter Mitchell had already recognised an alternative mechanism able to drive electron transfer from higher to lower potential redox compounds: redox bifurcation [26–28]. This mechanism requires the participation of a 2-electron redox compound that can be redox-tuned to perform either two consecutive single-electron transfers or an apparently concomitant 2-electron transition. In the latter type of reaction the endergonic "uphill" electron transfer can be energetically pulled by a strongly exergonic redox reaction if the sum of both reactions remains exergonic. A series of seminal articles during the last 3 years [25,29,30] have shown that this kind of redox trick, previously thought to only occur within the Q-cycle of the Rieske/cyt*b* complexes, is exploited by acetogens and methanogens to drive electrons up to the very reducing redox potentials needed to fuel reduction steps in the Wood–Ljungdahl pathway [25]. This mechanism requires 2-electron redox compounds which can shuttle between crossed-over and uncrossed midpoint potentials for the individual redox states (for details, see [31]. Quinones, methanophenazines, flavins and even transition metals such as Mo and W can do this job and others may soon join this list (such as pyrroloquinoline quinone, PQQ, or thiamine pyrophosphate, TPP). Since this mechanism is based on fundamental electrochemical principles rather than complicated enzymatic function, it is likely to be much more widespread than presently appreciated [31]. The works by Buckel's and Thauer's groups indeed seem to have removed a thought barrier and the implication of redox bifurcation as well as its reversal, confurcation, is now invoked in many different, energetically challenging electron transfer steps [32–35]. Detection of potential two electron carriers and the determination of their electrochemical properties will in fact allow a decision to be made whether a given uphill redox transition may indeed rely on bifurcation or instead feeds on the chemiosmotic membrane potential.

Table 1
Useful conversions between electrochemical parameters and Gibbs free energy.

Effective electrochemical potential E_h at pH 7 of a redox compound:
(1) $E_{h,7} = E_{m,7} + 59\ mV/n * \log([ox]/[red])$
with $E_{m,7}$ denoting the redox midpoint potential at pH 7
and n, number of transferred electrons
Electrochemical driving force for redox equilibrations
between a donor (D) and an acceptor (A) molecule:
(2) $\Delta E_h = E_h(A) - E_h(D)$
Gibbs free energy of redox reaction:
(3) $\Delta G = -nF\Delta E_h$
with n, number of transferred electrons and F, Faraday constant

3.3. Bioenergetic electron transfer chains; get all the energy you can

As is evident from Fig. 2, the nominal ΔE_m of Fe^{2+} oxidation by O_2 ($\Delta E_m = 300$–200 mV) is dwarfed by that calculated for the so-called Knallgas-reaction oxidising molecular hydrogen to reduce O_2 ($\Delta E_m = 1200$ mV). As a result, the number of protons translocated across the membrane for each electron transfer from Fe^{2+} to O_2 (see article by Ilbert and Bonnefoy in this issue [36]) is substantially lower than when electrons flow down the more than 1 Volt slope from H_2 to O_2. In molecular terms this translates into a lower number of "coupling sites", that is, H^+-translocating enzymes in chains exploiting low ΔE_m-substrate couples as compared to the high ΔE_m systems. In microbiological terms this spells "low yield" and frequently "slow growth", facts painfully known to everybody who has for example grown the iron-oxidiser *Acidothiobacillus ferrooxidans*.

Low ΔG-schemes, however, aren't restricted to the high potential regimes (as indicated in the rightmost column of Fig. 2). Organisms reducing oxidised sulphur compounds or CO_2 (leftmost columns) are even more energy-limited than Fe^{2+}-oxidisers but thrive in a completely different redox environment. In between these extremes,

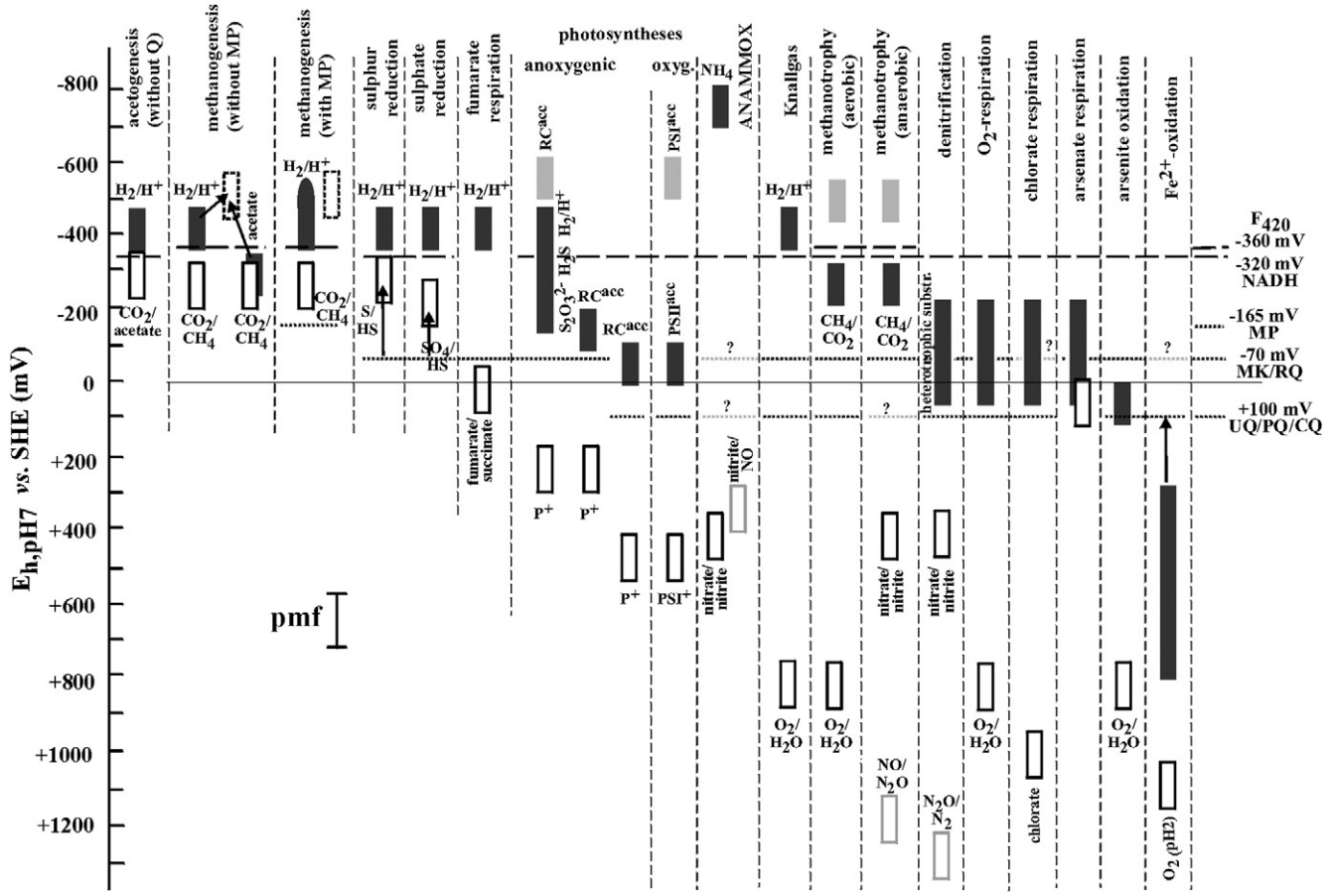

Fig. 2. Schematic representation of the electrochemical characteristics of a variety of diverse chemiosmotic systems. Reducing substrates are indicated as dark filled rectangles whereas the oxidising substrates are denoted by open rectangles. Boxes filled in lighter grey stand for reductants produced *via* a preceding initial oxidation of the environmental reductant. Analogously, boxes with light grey borders mark secondary oxidants. The vertically extended dark filled boxes centred at 0 mV comprise a range of different, mostly heterotrophic electron donors. The vertically extended box in the Fe^{2+} oxidation column emphasises the strongly variable potential of Fe^{2+} as a function of its counterion [144]. The dark filled rectangle of the electron donor in the third column is elongated towards more negative potentials to emphasise the more negative E_h (see formulae in Table 1) entailed by increased H_2 concentrations necessary for growth of these species. Dashed and dotted horizontal lines indicate $E_{m,pH7}$ values of NADH/F_{420} and diverse quinone types, respectively, as detailed in the right margin of the figure. Upwards arrows highlight energetically challenging electron transfer steps (see main text). The dotted rectangles represent low potential electron carriers in the methanogenetic pathways. The average value of the proton-motive-force (pmf) expressed in mV is indicated in the lower left corner of the figure for comparison with available redox energy in the diverse chains.

the E_m gap opens up in an almost continuous transition towards the highest ΔE_m systems represented by the Knallgas reaction, methanotrophy, denitrification and aerobic respiration.

Fig. 3A, C and D schematically visualises the growing complexity of chemiosmotic electron transfer chains with and without liposoluble and hydrosoluble carriers and/or pumping enzymes, resulting from, and made possible by, the increasing free energy content of electrochemical substrate couples. The number of coupling sites ranges from one (Fig. 3A) to at least 3 in Fig. 3D, or even higher as discussed in Section 3.3.4.

3.3.1. Bioenergetic systems lacking liposoluble hydrogen carriers

Methanogenesis and acetogenesis using H_2 and CO_2 as electron donor and acceptor, respectively, certainly are at the low energy edge for chemiosmosis. This fact is consistently pointed out in articles dealing with these two types of energy metabolism [25,37,38]. Their respective nominal ΔE_m values of 180 mV and 130 mV appear hardly sufficient to allow forward electron transfer towards CO_2 while still leaving enough energy to pump a proton (or sodium ion) to build up chemiosmotic potential. This energy poverty is probably at its acme in methanogens using acetate as electron donor ([39,40] and see Fig. 2, third column). Hence predictably, many acetogens and methanogens other than methanosarcinales contain only a single chemiosmotically coupling enzyme which in acetogens is an entity now referred to as the Rnf-complex [35,41–44] while in methanogens, it appears to be the enzyme methyltransferase (Mtr, [45]). Ech-type [NiFe] hydrogenases [46–48] may also perform redox-coupled H^+/Na^+-pumping reactions as Rnf and Mtr do. Fig. 3A represents the basic layout of such an ultra-short chemiosmotic electron transfer chain (for details on specific systems, see the article [35] by Buckel and Thauer in this issue).

3.3.2. Coupled proton and electron transport via liposoluble hydrogen shuttles

If more energy is contained in the electrochemical span of a given substrate couple, prokaryotes generally resort to membrane-soluble hydrogen carriers, in the form of small organic molecules such as quinones or methanophenazines. These mediate electron transport from a donor-oxidising to an acceptor-reducing enzyme (Fig. 3B). For simplicity, both true quinones (including mena-, ubi-, plasto- and caldariellaquinones) and methanophenazine will be abbreviated as Q in Fig. 3. These hydrogen carriers, co-transporting two protons and two electrons, are uncharged and their diffusion from one side of the membrane to the other therefore isn't influenced by the membrane-potential. Basic molecular architecture of the reducing and oxidising enzymes allows adjustment of the number of protons translocated per electron in the chain to the energy available in the redox couple. In order to maximally augment chemiosmotic membrane potential, Q reduction sites need to be located at the negative (N-), *i.e.* in most cases the cytoplasmic face of the membrane whereas

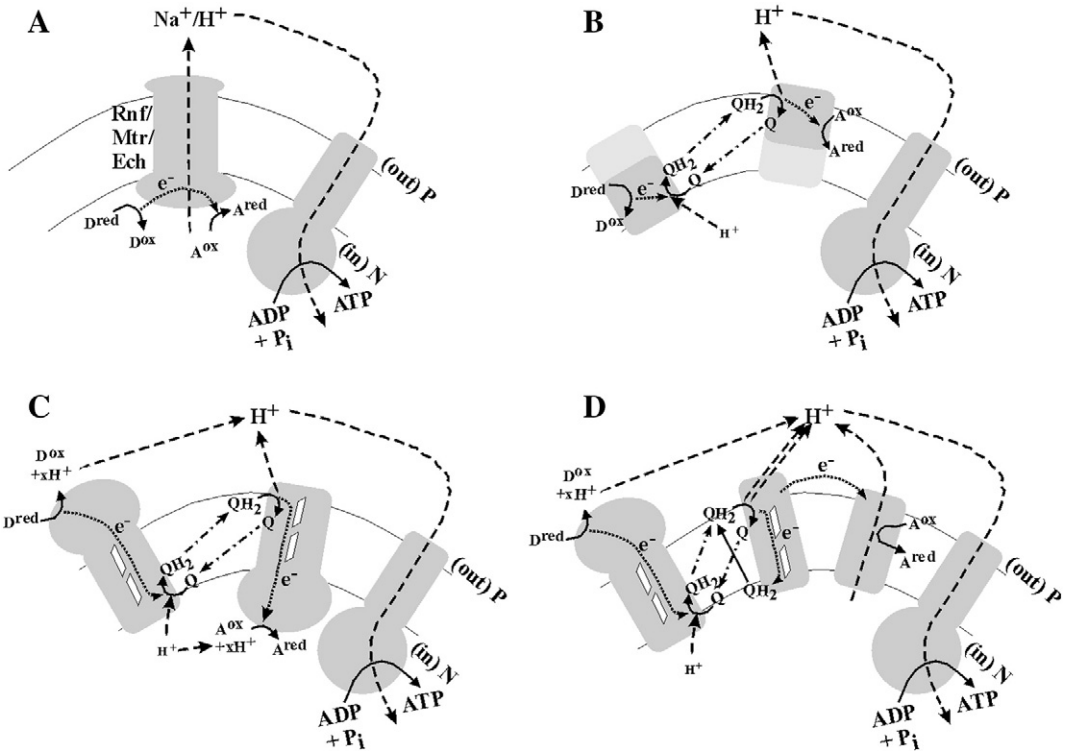

Fig. 3. Schematic representation of the increasing complexity of chemiosmotic electron transfer chains as a function of available substrate redox energy. (A) Shortest possible chain not involving liposoluble carriers. (B)–(D) Quinone-based chemiosmotic systems as discussed in the main text. Dashed and dotted arrows stand for proton and electron transfers, respectively, while dash-dotted arrows indicate diffusion of the liposoluble carriers.

QH_2 oxidation should occur on the opposite (P-) side. If the membrane proteins providing Q-reduction and QH_2-oxidation sites were only "scaffolds" imposing the abovementioned sidedness of quinone redox reactions, the electron donating and accepting substrates would necessarily react on the N- and P-sides, respectively. Such a layout would, however, raise a major efficiency problem. Due to charge compensation, the vast majority of substrate oxidations liberate protons whereas substrate reductions consume them. Thus, if the reducing substrate were to be oxidised at the N-side (liberating protons), this reaction would result in collapsing the chemiosmotic potential, just as the reduction of acceptor (and hence consumption of protons) on the P-side would do. The proton translocation linked to quinone redox reactions would thus be more or less cancelled out by the substrate conversions depending on the pK-values of substrate deprotonations. Life has overcome this chemiosmotic challenge by connecting substrate- and Q-reacting sites on opposite sides of the membrane by an electric wire (Fig. 3C) which in almost all cases takes the form of a membrane-integral protein piling up two heme groups edge-to-edge so that they span the membrane and allow electron transfer from one side to the other (see Section 6.3).

The case of the Methanosarcinales as compared to the previously mentioned methanogens nicely illustrates the impact of thermodynamics on the layout of bioenergetic chains. Methanosarcinales grow in environments containing H_2 levels superior to those typically encountered by other methanogens [49]. From the mass-action contribution to ΔG (see Table 1) it follows that the free energy contained in the H_2/CO_2 substrate couple increases with mounting concentrations of H_2. Indeed, whereas the remaining methanogens appear to harvest energy according to the scheme in Fig. 3A, Methanosarcinales operate bioenergetic electron transfer chains as shown in Fig. 3C, containing the hydrogen atom carrier metanophenazine (MP) and thereby adding a second coupling site. The donor H_2 is oxidised on the positive (*i.e.* outer) side of the membrane by a Group 1 [NiFe] hydrogenase and the resulting electrons are shuttled through the membrane *via* a cyt b^l-type membrane-integral protein (see Section 6.3) towards a MP-reducing site. MPH_2 is in turn oxidised at the cytochrome subunit of membrane-associated heterodisulfide reductase, the second coupling site of the chain. The resulting increased energy output allows Methanosarcinales to reach growth yields more than twice those of other methanogens but, as payoff, precludes them from thriving at low levels of H_2 [25].

3.3.3. Fine-tuning of chain architecture to meet thermodynamic constraints

Of course a quinone-based bioenergetic chain would ideally look like the scheme of Fig. 3C, if it weren't for thermodynamic limitations imposed by the substrate couples. In fact, in the scheme of Fig. 3C, the electrons shuttled across the membrane have to go energetically uphill against the chemiosmotic membrane potential. This implies that additional electrochemical energy is necessary to make the electron transport chain of Fig. 3C work efficiently in the forward direction, that is, oxidising the donor D and reducing the acceptor A rather than the reverse. Since for a single 1-electron transition, 120 mV of ΔE_m is required to roughly shift 90% to the product state (set $E_h(D) = E_h(A)$ and concentration ratios of 10 and 0.1 for [ox]/[red] of D and A, respectively in Table 1), we tend to take a ΔE_m of at least 100 mV as a rule of thumb for efficient forward electron transfer between substrates in sustained turnover. If electron wires as in Fig. 3C are involved, additional redox energy exceeding 150 mV for each passage of the electron against the membrane potential must be provided by the ultimate couple of redox substrates. Thus, in a substantial number of real-world bioenergetic chains, scheme in Fig. 3C cannot exist due to lack of sufficient redox energy. Examples for such situations are provided by the H_2/fumarate chain (see [50] or the reduction of several sulphur compounds by H_2 [51]. In such chains, one or several (for cases as discussed in Section 3.3.4) transmembrane electron transfer steps are sacrificed which has the effect of reducing the number of coupling sites from the theoretical

optimum of Fig. 3C to the thermodynamically allowable limit. Various molecular strategies for adjusting the number of coupling sites and proton/electron stoichiometries to the amount of redox energy contained in the substrate couples are discussed in a recent excellent review on this topic [52].

Numerous variations to the scheme of Fig. 3C are found in prokaryotes. These variations in most cases reflect an energy maximising compromise between the above described proton translocation through quinone/methanophenazine shuttling, proton uptake/release from redox conversions of individual substrates and the limitations imposed by thermodynamics.

3.3.4. Redox-substrate-breeding reactions

The redox conversion of the environmental substrate can, for particular substrates, generate further donor and acceptor molecules (the most reducing and oxidising of which are indicated by grey rectangles and open ones with grey borders, respectively, in Fig. 2). An example for such a donor is found in methane oxidation, which yields subsequently methanol and several tetrahydromethanopterins with C1-bodies in increasing oxidation states, all of which act as electron donors to either chemiosmotic chains or metabolic reductants. On the electron accepting side, a prominent case is provided by denitrification in which the reduction series proceeds from nitrate through nitrite, nitric oxide and nitrous oxide ultimately to N_2. The product of each reduction step serves as electron sink in the subsequent steps and all these sinks may thus be useful for chemiosmotic membrane potential build-up.

3.3.5. Towards even higher redox spans

As is evident from Fig. 2, for the case of the strongest donor/acceptor redox differences, the available electrochemical energy largely exceeds the chemiosmotic energy that can be harvested by bioenergetic chains of the Fig. 3C-type. Rather than simply dissipating this excess energy as heat, bioenergetic chains under these conditions employ independently or simultaneously two strategies. (i) The coupling enzymes pump extra protons *via* conformation-based mechanisms. Examples for such behaviour are heme-copper O_2 reductases (*e.g.* as represented by the acceptor-reducing enzyme in Fig. 3D) or Complex I which has three proton pumping subunits operating in parallel, all driven by the electron transfer from NAD(P)H to quinone [53]. (ii) An additional coupling enzyme is interposed between the donor-oxidising and the acceptor-reducing complexes (Fig. 3D). This additional enzyme in the majority of cases is the Rieske/cyt*b* complex (including Complex III, bc_1- and b_6f-type enzymes). The Q-cycle [26] catalysed by this enzyme exploits excess redox energy by rechanneling part of the electron flow from quinol oxidation back into the quinone pool and thereby substantially increases the energetic efficiency of the whole chain. Some of the authors of the present contribution have therefore chosen in previous publications to call the Rieske/cyt*b* complexes the "turbo chargers" of bioenergetic chains [9,54].

In recent years, a second enzyme possibly carrying out a comparable redox reaction has been described and called "Alternative Complex III" (ACIII, [55,56]). We cannot help noticing that ACIII is incommensurably more complex than the Rieske/cyt*b* complex (disregarding its eukaryotic versions) with respect to number of protein subunits, total molecular weight and number of redox cofactors (6 *c*-hemes and 4 cubane iron sulphur centres as compared to 3–4 hemes and 1 [2Fe2S] cluster in the Rieske/cyt*b* enzyme). ACIII therefore is metabolically much more costly (with respect to biosynthesis and the element Fe) than the Rieske/cyt*b* complex and it appears unlikely that ACIII is merely an "alternative" Complex III (see also [34]).

3.3.6. Is there a way to exploit larger ΔE_ms without using quinones?

Empirically, apart from those aceto- and methanogens that lack quinones or methanophenazines, all chemiosmotic chains observed in extant organisms use liposoluble hydrogen carriers as schematised in Fig. 3B–D. The small amount of redox energy contained in the H_2/CO_2 substrate couple appears to preclude these particular aceto- and methanogens from adopting the quinone-option. Indeed, the ubiquity of the Q-based chain seems to suggest that the lipophilic carrier principle is life's unique way to optimally use higher redox span substrate couples. However, the emerging picture [53] of the mechanism employed by Complex I indicates that this probably isn't the case. Complex I may indeed make three distinct proton pumping subunits analogous (or even homologous) to Rnf, Mtr or Ech function in parallel and synchronously, all driven by the redox transition from NAD(P)H to quinone. This alternative mechanism notwithstanding, it appears to us that the molecular simplicity of quinone-based proton translocation as compared to Complex I (dubbed "The most complex complex" [57]) and even more, its extreme versatility with respect to redox couples ([52] and see above), rationalises why life has invariably resorted to lipophilic carriers when sufficient substrate redox energy was present.

3.3.7. Thermodynamics sets the frame, evolution draws the picture

It is noteworthy that the energetic arguments that we have outlined above do not always lead to a unique optimal configuration for a given bioenergetic chain or even of parts of it. The example of nitrate reduction nicely illustrates that different outlines may still result in energetically viable systems. Since nitrate reduction consumes 2 protons, Fig. 3C would suggest that this reduction should occur in the cytoplasm. This is indeed how dissimilatory nitrate reduction is carried out by all members of the Bacteria characterised so far. However, nitrate is an anion and transporting environmental nitrate from the cell exterior to the cytoplasm goes against the polarity of the membrane potential and therefore consumes energy. According to all biochemical and bioinformatic data available so far [58–60], Archaea (with possibly a handful of exceptions; Schoepp-Cothenet et al., unpublished results) seem to perform nitrate reduction on the outer face of the membrane. They appear to make up for the counterproductive uptake of protons at this outer face by involving a Rieske/cyt*b* complex in electron transfer towards the nitrate reductase enzyme [59,60]. The additional proton translocated by the Rieske/cyt*b* complex together with the fact that no energy is spent for nitrate import render this substantially different architecture of a nitrate reducing bioenergetic chain apparently just as viable as the bacterial one.

3.3.8. How does Escherichia coli fit into all that?

Doesn't *E. coli* bioenergetics contradict this whole section? Several bioenergetic chains have indeed been characterised in this species which clearly do not harvest all the energy they can. The redox-loop mechanism connecting formate oxidation to reduction of nitrate [61,62] is a well-known example for *E. coli*'s spendthrift ways to convert energy. The almost 1 V of redox energy contained in this substrate couple is mostly wasted and not even a Rieske/cyt*b* complex is present in *E. coli* to allow an extra round of quinone-mediated proton translocation. A survey of organisms using such profligate bioenergetic chains indicates that this behaviour is a particularity of endosymbionts and parasites. To the best of our knowledge, no obligatorily free-living species has been described which doesn't go to the thermodynamic limit in exploiting available redox energy. It therefore seems likely that it is the energetic paradise the endosymbionts live in that allows them to use less-than-optimal chemiosmotic chains when dealing with their substrates. Nevertheless, we would argue that even in these cases thermodynamics is the dominant factor shaping the makeup of bioenergetic chains. If a large excess of redox substrates is available, it may be energetically more advantageous to have a lower number of coupling enzymes – the biosynthesis of which consumes ATP and chemical building blocks – to turn over at maximum speed than to maximise the number of coupling sites as would be favourable when redox resources are limited.

4. Availability of redox substrates through 4 billion years of life on Earth

The present day plethora of differing couples of redox substrates was likely not always available throughout life's history. A crucial parameter for hypotheses on the nature of ancestral chemiosmotic systems and their evolutionary expansion therefore lies in a reliable palaeogeochemical scenario for the variations of environmental redox compounds over the last 4 billion years.

The most solid set of data certainly is that pertaining to the levels of atmospheric O_2. Molecular oxygen appeared in the biosphere in appreciable amounts by about 2.3 billion years ago, a major palaeogeochemical transition termed the "Great Oxidation Event" (GOE), with possibly transitional "whiffs of oxygen" prior to the GOE [63,64]. Even if the atmosphere prior to the GOE was almost certainly not reducing as previously assumed [65], the absence of substantial amounts of the strong oxidant O_2 precluded moderately high potential ($E_m > 0$ mV) substrates from accumulating in their oxidised states. Not only respiratory chains ending at molecular oxygen but also those using substrates likely produced by high O_2-levels (such as selenate, chlorate, DMSO, TMAO or arsenate) are therefore unlikely candidates for fuelling ancient bioenergetic chains. The majority of molecular phylogenies reconstructed on redox enzymes converting such high potential substrates indeed show signs of evolutionarily late emergence [66–70]. Palaeogeochemical data with respect to another ecologically important redox substrate probably pre-dating O_2, sulphate, are still controversial [71].

By contrast, there is broad consensus that the electron donor H_2, as well as the acceptor redox compound CO_2, was abundant in certain locations even during the earliest eons of our planet [72–75]. As will be discussed in more detail below, this fact makes acetogenesis and methanogenesis likely candidates for very early types of energy (and carbon) metabolism.

Whereas the presence of the H_2/CO_2 couple of redox substrates appears beyond reasonable doubt, other electrochemical substrates have been proposed based on palaeogeochemical reasoning. Nitrogen oxides and nitrogen oxyanions, abiotically produced by electrical discharge and heat, have been put forward over 20 years ago [76] as ancestral sources of both bio-accessible nitrogen and energy. The interpretations of molecular phylogenies reconstructed for enzymes involved in redox conversions of nitrogen compounds are presently debated with some authors taking them to support early presence of nitrate and nitrite [77,78] while others propose alternative scenarios to rationalise the observed trees [79–81]. Since nitrogen oxides and nitrogen oxyanions are strongly oxidising redox compounds, their presence would allow energy converting chains with high ΔE_m to have existed prior to the rise of oxygen on Earth. The question of the presence or absence of these substrates is a crucial parameter in evaluating the likelihood of scenarios for energy metabolism on the early Earth. Palaeogeochemical evidences pertaining to this problem are thus badly needed. On the basis of molecular phylogenies, the authors of this article presently tend towards a model counting nitrate and nitrite into the repertoires of early Earth redox substrates. For discordant opinions, see the articles by Simon and Klotz [80] and by Chen and Strous [81] in this issue.

It is noteworthy that the expedition to the submarine hydrothermal "Lost City" field [82] has provided indications for a putative further redox substrate in vestiges of primordial life: methane. Vent effluents were indeed found to contain millimolar concentrations of abiotically formed CH_4, almost certainly produced from H_2 and CO_2 during the serpentinisation process [83–86].

5. Quinones and methanophenazines, crucial elements of almost all bioenergetic chains

Apart from methano- and acetogens other than Methanosarcinales, no species have been discovered so far which do not implicate liposoluble hydrogen carriers, *i.e.* quinones or methanophenazines, in their bioenergetic electron transfer chains. As indicated by the dotted horizontal lines in Fig. 2, different kinds of chemiosmotic systems use different types of liposoluble H_2 carriers. Several bioenergetic chains appear to be obligatorily connected to specific types, such as methanogenesis of Methanosarcinales to methanophenazine, sulphate reduction to menaquinone or oxygenic photosynthesis to plastoquinone. The parent species of these chains correspondingly only use these molecules for their liposoluble pools whereas other organisms are, for example, able to switch between mena- and ubi- or rhodo- and ubiquinones. The principal parameter distinguishing the different kinds of quinones is their redox midpoint potential. Mena- and rhodoquinones are "low-potential' carriers both featuring E_m-values close to −70 mV [87–89]. Opposite to this low potential pair stands the group of high potential quinones so far represented by ubi-, plasto- and the crenarchaeal caldariellaquinone [90] with an E_m-value of about +100 mV. Methanophenazine constitutes the third, most reducing electrochemical variant with E_m of −165 mV [91]. Six chemically different liposoluble hydrogen carriers thus fall into only 3 electrochemical groups raising the questions of (a) why bioenergetic chains may need hydrogen carriers with differing E_m values in the first place and (b) why different species bother using chemically different molecules (with differing biosynthetic pathways) to arrive at functional entities doing the same job. As already discussed in 1995 [92], a possible answer to the second question, *i.e.* concerning the surplus of chemical solutions for the 3 distinct redox regimes is suggested by the distribution of the mentioned types of liposoluble carriers on the phylogenetic tree of species (Fig. 4). This distribution strongly indicates that menaquinones are both the dominant and the ancestral type of liposoluble H_2 carrier in prokaryotes. The higher and lower potential redox species appear to have emerged separately in 4 different locations on the tree, that is, ubiquinone in the common ancestor of α-, β- and γ-proteobacteria [93], caldariellaquinone in Sulfolobales [90], plastoquinone in the ancestor of the cyanobacteria and methanophenazine in Methanosarcinales [25]. Rhodoquinone seems to have appeared in selected α- and β-proteobacterial species, intriguingly in selected species which likely have lost the ability to synthesise menaquinone. The excess of 3 chemical forms of high potential quinones is thus likely to be the result of independent evolutionary innovations of new carriers to replace or supplement the ancestral menaquinone. This scenario suggests the presence of an evolutionary selection pressure at some time in the evolutionary past to switch from the −70 mV to the +100 mV redox regime. This conspicuous emergence of "islands" of high potential quinones from the low potential menaquinone-"sea" (Fig. 4) has in fact been argued to reflect independent adaptations to a higher environmental redox state induced by the establishment of substantial O_2 levels following the GOE [92,93]. Menaquinones are indeed readily oxidised by molecular oxygen possibly entailing substantial leakage of electrons from the respective chemiosmotic chains and ROS production.

The late appearance of the low potential rhodoquinone in α- and β-proteobacteria which do not synthesise menaquinone anymore, may have been driven by the need to rescue low potential pathways after mutational loss of menaquinone biosynthesis genes to recolonise anaerobic habitats. Alternatively, the fact that synthesising a low potential rhodoquinone through few or even only a single additional step(s) from ubiquinone [94] is energetically less costly than maintaining a full-fledged independent pathway for menaquinone biosynthesis may have prompted organisms to do away with menaquinone as their low potential liposoluble carrier.

Coming up with an evolutionary scenario for the emergence of methanophenazine is less straightforward. Fig. 4 seems to indicate that the emergence of methanophenazine might have provided a means for Methanosarcinales to connect up the very low redox span from H_2/H^+ to CO_2/methane to bioenergetic chains operating with liposoluble H_2 carriers when enough ΔG is available, *i.e.* when H_2 pressures are high. However, the bulk of methanogens other than

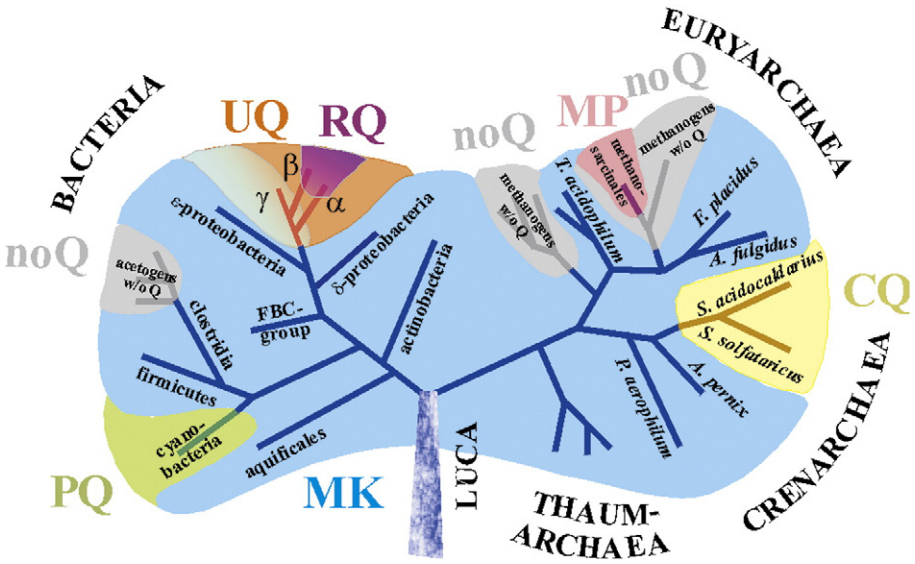

Fig. 4. Distribution of different chemical types of quinones and methanophenazine on a schematic and simplified version of the phylogenetic tree of prokaryotes. The archaeal part of the tree is based on a recent re-assessment including the newly defined phylum of the thaumarchaea [145]. On the bacterial side, only consensus-branching orders (as encountered in almost all published phylogenetic trees of the bacterial domain) are included. Aquificales and Actinobacteria feature as the most basal phyla, cyanobacteria are shown to cluster with low-GC Gram-positive bacteria (including Clostridia). The branching orders of the proteobacterial subgroups are basically unambiguous. The only bacterial phylum on this tree the positioning of which is somewhat controversial is the FBC-group. Since species from this latter phylum appear to exclusively contain menaquinones, their exact branching node is of no importance to the inferences drawn in the text. Colour-coding is as indicated in the figure. The smooth transitions in colours within the proteobacterial subgroups are meant to indicate that more than one type of quinone is present in the respective species, *i.e.* mena- and ubiquinone in γ-proteobacteria or ubi- and rhodoquinone in certain α- and β-proteobacteria.

Methanosarcinales contain neither quinones nor quinone-oxidising or reducing enzymes. Either these enzymes have been re-imported laterally into Methanosarcinales or the notion that methanophenazine-containing Methanosarcinales are derived from methanogens without methanophenazine is erroneous. In that case, one might speculate that Methanosarcinales represent the ancestral scheme of methanogens and that methanogenesis without methanophenazine is the derived trait imposed by colonisation of less hydrogen-rich habitats. This latter model admittedly requires a large number of independent losses of the methanophenazine-based electron transfer chains. In any case, the evolutionary relationship between methanogens with and without methanophenazine remains poorly understood and represents an exciting field for future research.

6. The redox protein construction kit; 10 years later

As indicated in Fig. 2, a wide variety of electrochemical substrates is exploited by life for the sake of harvesting energy. The varying amounts of energy available in specific substrate couples as well as the diversity of redox ranges (see Fig. 2) result in substantially differing layouts of the corresponding bioenergetic chains (Fig. 3, see also [52]). The mentioned example of nitrate reduction illustrates that even for a given redox substrate, different species have come up with different solutions to the challenge of harvesting the available energy.

Surprisingly, the multitude of the above described reactions is catalysed by only a few individual enzyme architectures. Sequence information in the first place and then the increasingly available 3D structures of bioenergetic enzymes have led to a stunning conclusion: life uses a very limited basic set of protein building blocks to construct the enzymes that spawn the immense variety of bioenergetic electron transfer chains. Ten years ago, some of us presented an early snapshot of the emerging picture of this "redox enzyme construction kit" [95]. As of today, the number of elements in the construction kit has grown only marginally while many more enzymes using elements from the kit have been described and substantially more details with respect to structure and function of individual building blocks are now at our disposal. In the following section we will present a short update on some of the elements previously discussed in 2002 and mention a few newcomers to the kit. Particular attention will be given to the dissimilar but mutually cooperative roles of the diverse units allowing the assembling of bioenergetic chains which conform to the thermodynamic constraints as outlined in Section 3. The kit in fact contains proteins acting as catalytic subunits as well as electron wire units or scaffolds for quinone reaction sites. The following sections will first deal with catalytic subunits and then move on to electron wires and Q-binding subunits.

6.1. The molybdopterin/tungstopterin-protein; the catalytic workhorse of bioenergetics

An ever increasing number of enzymes containing a catalytic subunit following the basic architecture depicted in Fig. 5A have been discovered and characterised during the last two decades [96]. Initially, this group of proteins was referred to as the DMSO reductase superfamily after its first well-studied representative. It soon became clear that the enzyme DMSO reductase was in fact relatively atypical with respect to its overall structure and cofactor composition. The name Complex-Iron–Sulphur–Molybdenum- (CISM-) superfamily was therefore coined a few years ago [68]. Despite its shortcoming of neglecting the fact that several members of the superfamily carry a tungsten atom rather than molybdenum in their catalytic site, this designation has the merit of emphasising the simultaneous presence of a molybdenum centre (ligated by two pterin molecules and – optionally – a protein ligand) and a cubane ([3Fe–4S] or [4Fe–4S]) iron sulphur cluster (see below). Only very few selected cases of genuine DMSO reductases lack the iron sulphur centre binding domain and all phylogenetic evidence suggests that (i) the loss of a domain was a relatively late event and (ii) the common ancestor of the whole superfamily almost certainly contained both the Mo/W-atom and the cubane iron sulphur centre [68,70].

The most conspicuous property of CISM-proteins as compared to other building blocks is their extreme versatility for substrates and redox regimes. CISM-enzymes convert molecules ranging from the simple inorganic molecule CO_2 to molecules considered xenobiotics

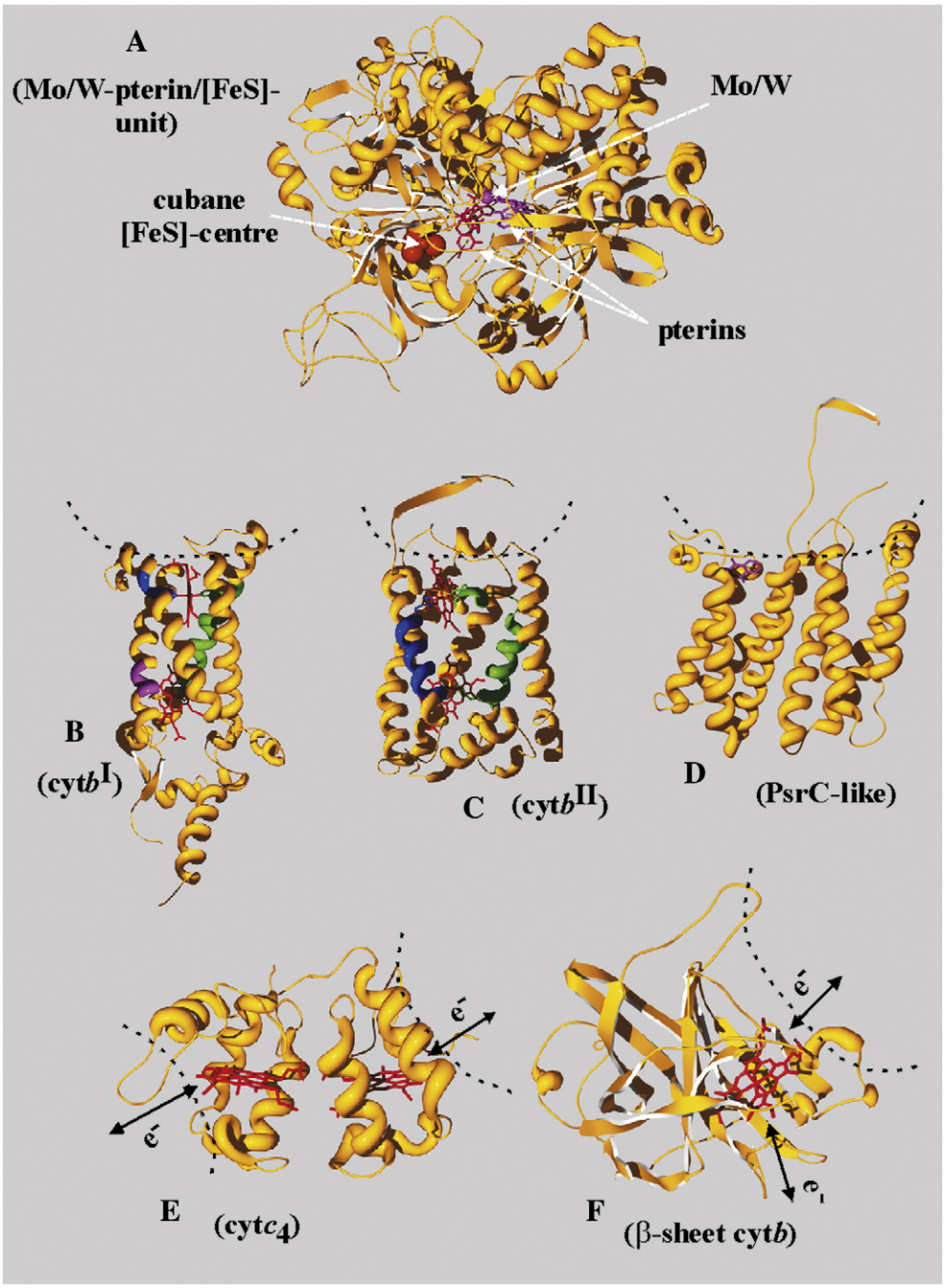

Fig. 5. 3D-structures of selected members of the "redox enzyme construction kit". (A): The Mo/W-pterin catalytic subunit as exemplified by the corresponding subunit (pdb-entry 1G8Ja) of the enzyme arsenite oxidase. (B) and (C): Cytochromes b^{I} (pdb-entry 1KQGc) and b^{II} (pdb-entry 1Q16c). Sequence stretches containing heme ligands are coloured in blue, green and magenta. (D): The PsrC-type unit (pdb-entry 2VPWc) featuring the transiently bound quinone molecule (in magenta). (E): Cytochrome c_4 (pdb-entry 1H1O). (F): The β-sheet *b*-type cytochrome (pdb-entry 2IVFc) as discussed in the main text. For (B)–(F), the dashed line represents the approximate position of the neighbouring subunit in the enzyme complex. Arrows in (E) and (F) indicate likely electron transfer routes. Structures were visualised using Deepview [146] and POV-Ray.
Persistence of Vision Pty. Ltd. 2004, POV Raytracer (Version 3.6) [Computer software]. Retrieved from http://www.povray.org/download/.

such as ethylbenzene [97], chlorate [98] and acetylene [99]. The redox midpoint potentials of these substrates range from close to −500 mV (CO_2/methylmethanofuran) to +800 mV (chlorate/chlorite) and CISM-enzymes therefore catalyse redox reactions spanning more than 1 V, a feat to our knowledge achieved by no other bioenergetic enzyme superfamily. Molecular phylogenies of the CISM subunit [68–70] indicate that several CISM enzymes likely were present in LUCA. Life therefore appears to have exploited the remarkable catalytic versatility of the CISM protein since its very earliest days.

It is likely that the key to this versatility lies in the electrochemical plasticity of the molybdenum and tungsten atoms. Both Mo and W are trans-iron elements with valence electrons in the 4d and 5d shells, respectively, *i.e.* with a substantially weaker electron–nucleus interaction than for example in iron, copper or still smaller atoms such as carbon, oxygen or nitrogen. The two physiologically relevant redox transitions, that is, Mo/W(IV)–Mo/W(V) and Mo/W(V)–Mo/W(VI), therefore are much more strongly modulated by the exogenous electrostatic environment of these ions than in the cases of

iron and copper. The two redox transitions of molybdenum have even been shown to exist both in the uncrossed (*i.e.* featuring two consecutive 1–e^- transitions with a stable Mo(V) state, [100–102]) and in the crossed-over regime (with a direct 2-electron transition between the Mo(IV) and the Mo(VI) states, [103]), a property usually only observed in the conjugated molecular orbitals of cyclic organics such as quinones or flavins. Notably, the possibility of operating either in the uncrossed or the crossed-over redox regimes depending on the protein environment is quintessential for the functioning of crucial bioenergetic processes [28,35] as discussed above in Section 3.2, since it is the crossed-over state that allows them to achieve energetically uphill redox reactions *via* redox bifurcation [31]. From a bioenergetic point of view, molybdenum and tungsten centres corresponding to the active sites in CISM proteins can be considered as the inorganic equivalents of quinones and flavins in performing these peculiar redox exploits. These transition metals were part of the inorganic repertoire of the early Earth whereas the cyclic organics almost certainly are a product of nascent life [104]. Mo and W may therefore have performed all the redox bi- and confurcating functions that helped life to emerge, perhaps initially as an $Fe^{II}{}_5Mo_3^{VI/IV}S_{14}^{0/2-}$ cluster, while part of these functions have later been taken over by the mentioned organic molecules [105,106]. The ensemble of the unique redox properties of Mo and W thus rationalise the fact that the CISM protein stands out as life's most versatile bioenergetic enzyme.

6.2. Disulfide-related redox reactions by HdrD-like proteins; a newly emerging member of the redox kit

Bioenergetic enzymes involved in methanogenesis have in the past almost appeared as belonging to life from another planet [107]. Worse, the entire bioenergetic chains of methanogens looked fundamentally different from almost all characterised types of the wide variety of energy converting systems in prokaryotes. As detailed in Sections 3 and 5, an extreme redox and energy regime are imposed by the substrate couples and may have resulted in the particular architecture of these enzymes. However, over the past 10 years it became apparent that several enzyme complexes and individual protein subunits thereof are common to methanogenesis and respiratory chains. Prominent examples are [NiFe]-hydrogenases [47], ATPases [38] or the *b*-type cytochromes discussed below (see Section 6.3). Intriguingly, an enzyme almost emblematically associated with methanogenesis, *i.e.* heterodisulphide reductase (Hdr), now appears to be made up from subunits following the construction kit principle. In particular, the catalytic subunit (HdrD) performing the reductive cleavage of the disulphide bond between coenzyme M and coenzyme B, appears to be present in enzymes from species which clearly do not perform methanogenesis [25,33,50,51]. Although the specific catalytic role of these enzymes has not yet been definitively demonstrated, an involvement in disulphide redox chemistry is frequently proposed [33,50].

No 3D-structure of this catalytic subunit is available so far. Spectroscopic characterisations and sequence analyses/comparisons suggest that the subunit features, in addition to two conventional [4Fe–4S] centres, one or two (depending on the enzyme under consideration) copies of a very particular, probably cubane-type iron sulphur cluster identified by a characteristic sequence motif involving five cysteine residues [108]. This unique "5C"-cluster likely represents the catalytic site of the protein. To our minds, a better understanding of the HdrD protein and of the distribution as well as the functional role of its homologs will substantially further our comprehension of the links between methanogenesis and the remaining types of bioenergetic metabolisms.

6.3. Cytb^I and b^{II}; transmembrane electron wires connecting substrate conversions to the quinone pool

The two distinct types of transmembrane diheme cytochromes *b*, cytb^I and cytb^{II}, represent redox kit units "par excellence" as already discussed in [95]. They are part of enzymes as diverse as formate dehydrogenases, many Group 1 [NiFe] hydrogenases, membrane-bound nitrate reductases [95], heterodisulphide reductases [25], the Qmo-complex [33] and likely many more to come. In the 2002 version of the redox kit, only cytb^I's structure had been solved and hypotheses as to the structure of cytb^{II} and its integration into enzyme complexes had to rely on amino acid-based prediction methods. By now, both structures are available and have been compared, materialising the general picture previously suspected: Cytb^I usually contains 4 TM helices and the two heme groups are ligated by histidine residues on TM1 (1 His), TM2 (1 His) and TM4 (2 His). A 3D structure for a member of the family has been obtained in formate dehydrogenase from *E. coli* [109] (Fig. 5B). Cytb^{II}, shown in Fig. 5C and exemplified by the structure of the nitrate reductase enzyme [62], features 5 TM helices and the heme ligands are located on TM2 (2 His) and TM5 (2 His). It is unclear why there are two structurally distinct membrane-integral *b*-type cytochromes for the purpose of connecting the quinone pool to the membrane-extrinsic part of the enzymes. Group 1 hydrogenases even have recruited either cytb^I or cytb^{II} in different phylogenetic clades [50]. The observation that cytb^I appears to mainly serve as quinone-reducing and cytb^{II} as quinol-oxidising element might rationalise this duality but characterisations of further systems might, by the same token, quickly render this apparent correlation obsolete.

6.4. The PsrC/NrfD-family; a link between the quinone pool and substrate conversion skipping transmembrane electron transfer

As discussed in Section 3.3.3, insufficient ΔE_m of specific redox substrate couples does not allow the corresponding bioenergetic chain to link two (or more) uphill transmembrane electron transfer steps to substrate redox reactions (scheme of Fig. 3C). The PsrC/NrfD structural unit (Fig. 5D) represents one of the solutions employed by chemiosmotic chains to extract or inject electrons to or from quinones for substrate conversions under such energy-restricted conditions. Sequence homologies indicate that this protein takes part in several diverse enzyme complexes, such as polysulphide reductase, tetrathionate reductase, arsenate reductase [52,69] or a specific subclade of Group 1 [NiFe] hydrogenases [50]. Biochemical evidences suggested the PsrC/NrfD-type proteins to be devoid of electron transfer components such as hemes or [FeS]-centres. All the mentioned enzymes function in electron transfer chains with low ΔE_m, in keeping with the notion of insufficient redox driving force for fulfilling a complete Fig. 3C scheme.

The crystal structure of polysulphide reductase [110] provided the first 3-dimensional picture of this redox kit element (Fig. 5D). As expected, the almost completely membrane-integral PsrC protein was found devoid of redox cofactors. A quinone molecule is seen in electron transfer distance to the outermost centre of a chain of cubane iron-sulphur clusters (harboured by a redox construction kit member named "tetracubane iron–sulphur protein" in [96]) leading up to the catalytic subunit. It is noteworthy that from the X-ray structure of PsrC, Jormakka et al. [110] proposed a hypothetical mechanism allowing the subunit to translocate a proton by a conformational mechanism. If this hypothesis should bear out, PsrC/NrfD-type units may be more than just furnishing a protein scaffold for localising quinone reaction sites.

6.5. The cymA-family; an extra-membranous unit connecting soluble electron transfer to the quinone pool

Bioenergetic electron transfer schemes involving soluble electron carriers on the P-side of the membrane (*e.g.* see Fig. 3D) require interfaces connecting electron transfer in the soluble compartments to redox reactions of the liposoluble hydrogen carriers. Traditionally, it has been tacitly assumed that this task was fulfilled by the Rieske/cyt*b* complexes and, since very recently, "Alternative Complex III" was

discussed as carrying out an analogous function. However, as discussed in Section 3.3.5, involvement of the Rieske/cyt*b* complex necessitates a sufficiently high ΔG in the redox substrate couple to enable the pumping of an extra proton which is an intrinsic and obligatory feature of this enzyme. As suggested by the large redox span of ACIII's internal electron transfer chain (see [34]), also this enzyme may be able to translocate an extra proton across the membrane. However, the ΔG-poor cases of bioenergetic chains of Fig. 2, which still use lipophilic and hydrosoluble electron carriers, need a means to couple hydrophobic to hydrophilic electron transfer without draining too much energy. In many cases this means is provided by a member of the so-called cymA family of tetraheme *c*-type cytochromes.

No structure is yet available of a cymA-type cytochrome but its amino acid sequence predicts a soluble *c*-heme domain anchored to the membrane *via* an N-terminal TM-helix. Electrochemically, the four hemes fall into high and low potential pairs with E_ms in general below and above the potential of the physiological quinone, respectively. Quinol oxidation or quinone reduction only involves release or uptake of the protons on the quinones. No additional protons are pumped across the membrane and no extra redox energy is thus required.

6.6. Hydrophilic electron wire units

Many bioenergetic enzymes connect their substrate-reacting catalytic subunits to their electron entry/exit proteins through wire-type additional subunits. By wire-type subunit we refer to proteins that harbour several cofactors, arranged in a roughly linear manner with the only (so far) recognised function of transferring electrons from an entry to an exit point of the protein subunit.

The most prominent representative of this functional class of proteins is the tetracubane iron sulphur subunit featuring [4Fe–4S] and [3Fe–4S] clusters in a spatially roughly linear row as discussed in [95]. Another type of protein harbours two cubane clusters and is traditionally called "bacterial ferredoxin". In addition to its role as soluble electron shuttle protein, it occurs as a fixed subunit in several enzymes such as RCI-type photosynthetic reaction centres or certain Group 3 [NiFe] hydrogenases [111,112].

Among heme proteins of the wire-type are the diheme c_4-type cytochromes (Fig. 5E). They have two heme groups with a solvent exposed heme edge each and their opposite edges approach each other in standard electron transfer distances. Correspondingly, they occur both in transient electron transfer supercomplexes [112,113] and in stable enzyme assemblies such as specific Rieske/cyt*b* complexes [114,115] or O_2-reductases.

The hydrophilic β-sheet *b*-type cytochrome subunit represents an only recently recognised element of the construction kit. The heme group in this class of proteins features a high redox potential of >300 mV. A representative structure is available in the C-subunit of ethylbenzene dehydrogenase (Fig. 5F). This protein has furthermore been found in archaeal nitrate reductases [58], selenate reductases [102], DMS-dehydrogenases [101] or chlorate reductases [98] and is likely a subunit of selected archaeal Rieske/cyt*b* complexes [116]. A wire function in this single-redox-centre protein is achieved by exposing two distinct heme edges at the protein surface, reminiscent of the case of cytochromes c_1 and *f* [115].

7. Towards an elucidation of the evolutionary history and ultimately the common ancestor of bioenergetic chains

The multitude of differing bioenergetic chains using a variety of substrate redox couples (Fig. 2) very likely reflects diversification from a much more restricted basic set of chains or even from a single "founding" bioenergetic system using a unique ancestral couple of redox substrates. This diversification through 4 billion years of Darwinian evolution eventually allowed life to colonise all habitats on Earth featuring sufficient redox disequilibria or sunlight exposure. Understanding the evolutionary expansion of energy metabolism towards substrate pluripotency is thus the key to answering the question of how an inanimate Earth could have been transformed into the Gaia-system [117] of our present planet.

At the same time, basic thermodynamics require energy metabolism to be the very founding principle of life itself as increasingly pointed out during recent years [2,118] and as detailed in the article by Branscomb and Russell in this issue [1]. Following this argument, it seems inevitable to us that the origin of life must have occurred according to an "energy-metabolism-first" scheme. An important task therefore consists in weeding out more recently evolved mechanisms in the bioenergetic diversity and thereby to increasingly restrict the number of potential ancestral energy converting systems. This may ultimately lead to a minimal set representing the probable energy metabolism(s) at the origin of life.

It is noteworthy that plausible models for energy metabolisms at the origin of life have been developed which differ fundamentally from bioenergetic principles used by extant life [119–121]. This of course does not necessarily make them wrong but it implies the need for a total remake of energy capture between life's origins and the LUCA. Since herein we only deal with the common core and the roots of real-life, *i.e.* "observable" bioenergetic mechanisms, these alternative scenarios will not be further discussed in the context of this article. We cannot help noting, though, that the Occam's razor principle to our mind favours searching the likely roots of enthalpy capture at life's ultimate origin in the ensemble of what is actually out there in extant life over *ab initio* hypothetic pathways.

Two main approaches are typically employed in attempts at inferring the evolutionary history of chemiosmotic systems.

7.1. Geochemical evidences for the presence or absence of certain redox substrates and metal catalysts

As detailed in Section 4, palaeogeochemical evidences accumulated during the last few decades, if controversial in some cases, disqualify certain redox substrates and, as a consequence, the corresponding bioenergetic chains during certain eons of Earth's history. The most prominent example is provided by molecular oxygen which likely wasn't present beyond negligible levels before 2.5 billion years ago ([122], though for discordant voices, see [123]). The resulting lower environmental redox state prior to the GOE, as compared to present day conditions, likely entailed the absence of oxidised states of several other substrates with moderately high redox potentials. As a result these substrates were likely present predominantly in their reduced states excluding a role as terminal electron sinks. As discussed above, chlorate, arsenate or fumarate and further cases not addressed in Fig. 2 such as DMSO, TMAO or selenate, are unlikely substrates for early bioenergetics.

The lower redox state of the Archaean was furthermore proposed to have resulted in a virtual bio-unavailability of the metal catalysts copper (Cu) and molybdenum (Mo) [124]. Both these two metals are ubiquitous in and essential to almost all extant life. Availability increases of more than 10 orders of magnitude for Cu and 2 orders of magnitude for Mo going from the Archaean to the Proterozoic era have been suggested [64]. In Section 7.2, these predictions will be confronted with presently available evidences from molecular phylogeny.

Still, strongly oxidising molecules other than molecular oxygen, such as nitrogen oxides and nitrogen oxyanions, may have been present due to abundant formation in the CO_2/N_2 atmosphere through direct volcanic exhalation of NO augmented by electrical discharge [77]. The presence of strongly oxidising electron sinks in the early Archaean is therefore entirely possible [75,78,125]. The time of emergence of another ecologically important, although less strongly oxidising, terminal acceptor, sulphate, has remained controversial over the last 10 years [71]. It seems likely to us that palaeogeochemical and molecular studies on

sulphur metabolism will substantially influence future scenarios on the evolution of bioenergetic systems (see for example [126]).

Even if several substrates can thus be discarded as having served for ancient energy metabolism, others remain the matters of heated debate. The only undisputed couple of redox substrates presently appear to be molecular hydrogen as electron donor and carbon dioxide as acceptor, that is, the substrates of chemiosmotic chains represented in the three leftmost columns in Fig. 2.

7.2. Molecular phylogenies of bioenergetic enzymes

Sequence-based genealogies of bioenergetic enzymes potentially provide information concerning the evolutionary history thereof and, by inference, that of the correlated energy metabolisms and underlying redox substrates. Such information is mainly gathered from the comparison of molecular phylogeny of a particular enzyme to that of the parent species. Pervasive disagreements between these two phylogenies indicate dissemination essentially *via* horizontal gene transfer whereas a far-going congruence is likely to be observed for cases which have been mainly transmitted vertically through species lineages. Since the tree of species by definition has its root in LUCA, conclusions on the ancestry of enzymes in general fall into one of two categories, *i.e.* (a) presence of the considered enzyme already in LUCA or (b) its post-LUCA emergence. In favourable cases, post-LUCA emergence may be located more precisely on the tree of species [93]. Likewise, a few examples allow glimpses into details of pre-LUCA evolution such as a divergence of a specific enzyme into specialised subfamilies already prior to the Archaea/Bacteria divergence [70,111,112,127].

Since LUCA presently appears as an already quite sophisticated entity, a considerable time lapse likely exists between life's origin and its manifestation in the LUCA. This period is essentially inaccessible to molecular phylogeny and has consequently been dubbed the "dark ages" of life's evolutionary history [128]. Palaeogeochemical evidence by contrast, is not constrained by the LUCA-barrier and thus presently represents our only torch able to shine streaks of light into these dark recesses of time.

Although quite successful in determining evolutionary relationships between relatively recently diverged enzymes, molecular phylogeny is pushed to its limits when trying to resolve genealogies in deep evolutionary times, back to the LUCA. Many of the protein subunits present in bioenergetic enzymes are just too small and thus contain too few informative sites to allow reconstruction of reliable phylogenies over all domains of life. Nevertheless, we consider that by selecting sufficiently large proteins and by adding a maximum of contextual data one can arrive at phylogenetic trees with substantial levels of reliability and to deduce possible evolutionary scenarios from these trees, always being aware that phylogenetic results are probabilities and not certainties. Contextual information which to our minds permits us to substantially solidify the very fundamentals of phylogenetic reconstruction, *i.e.* the underlying multiple sequence alignments, can be gathered from structural and functional studies of the respective enzymes. If 3D-structures of a given protein from phylogenetically distant members of a family or superfamily are available, automated multiple sequence alignments can be refined by the results of structural alignments [70,129]. In the absence of an extended structural data set, intimate knowledge of functionally crucial sequence residues can help to eliminate obvious misalignments produced by automated alignment procedures which become overtaxed when the attempt is made to align phylogenetically distant and hence extremely divergent sequences [70]. In our experience, detailed knowledge of the peculiarities of a given enzyme furthermore allows detection of misannotations in genomes and thus eliminates false orthologs, a prerequisite for meaningful interpretations of the resulting phylogenies [69,130,131].

It is noteworthy that the very existence of the redox protein construction kit, *i.e.* the occurrence of certain protein units in a variety of enzymes strongly enhances the interest of molecular phylogeny for deducing the enzyme repertoire of LUCA. A phylogenetic tree of a given subunit from one enzyme only, even if it shows a perfect Archaea/Bacteria dichotomy, does not necessarily imply its presence in the LUCA since the position of the root remains speculative. However, the composite phylogeny of a protein subunit existing in several distinct enzymes permits mutual rooting of the individual subfamilies and thus enhances the reliability of pre-/post-LUCA inferences (as discussed in [60]).

Numerous enzyme phylogenies have been reconstructed over the course of the last decade. The vast majority of these phylogenies agree on the observation that Cu-containing enzymes appear comparatively late in evolutionary history (for discussions, see [60,80]) and most likely not before the accumulation of O_2 in the environment. For the case of Cu, results from molecular phylogeny are thus by and large in agreement with palaeogeochemical reasoning as mentioned in Section 7.1. The same isn't true with respect to molybdenum. Phylogenies of the Mo-pterin enzyme superfamily strongly support the presence of the enzyme in the LUCA. Palaeogeochemical rationalisations of this finding have been discussed in detail recently [70].

With respect to specific substrates, many enzymes corroborate the geochemical predictions outlined above. Potential terminal electron acceptors with moderately high redox potentials (see [131] for a detailed discussion of the case of arsenate) seem to have appeared in the biosphere only after the rise of oxygen, that is, substantially after the Archaea/Bacteria divergence. By contrast, certain enzymes converting H_2 or CO_2 indeed appear to have been part of the bioenergetic inventory of the LUCA [111,127,132]. Intriguingly, however, several specific enzymes catalysing reactions with high potential redox substrates or cofactors also look to have existed in the LUCA (see Section 4. and [60,70,133,134]).

Fraught with difficulties as the phylogenetic approach may be, we consider that it nevertheless holds promise for guiding our thinking about the evolutionary pathway of bioenergetic mechanisms from extant organisms back to the LUCA and thus as close as this approach can take us to the ancestral mechanism(s) of energy conversion at the origin of life.

7.3. Are we there yet?

In the literature trying to discern the most ancient bioenergetic pathway, a strong predictive weight is granted to the attributes "simple" and "energy poor" [30,35,37–39,44,118]. The simplicity principle is generally invoked both with respect to the pathway as a whole and to the involved individual enzymes. The fact that the Wood–Ljungdahl pathway (found in acetogenesis and methanogenesis) integrates both energy conserving and CO_2-fixing, that is, biomass-producing functions is often taken as a strong argument in favour of its deep ancestry [2,118,135]. The relative simplicity of the coupling enzymes (see Fig. 3A), Mtr in methanogenesis [45] and, as recently shown [40,43], of the Rnf complex in some acetogens, gives further credence to this notion.

As is obvious from Fig. 2, acetogenesis and methanogenesis from H_2 and CO_2 (and, even more so, from acetate and CO_2) are indeed much worse off than any other bioenergetic system with respect to electrochemical energy availability in their substrate couples: "Acetogens clearly live at the thermodynamic limit of life" [37].

The abovementioned arguments together with the re-evaluation of the atmospheric composition on the primitive Earth (*i.e.* stipulating high pressures of CO_2) and the recognition of seafloor hydrothermal vents (emanating high amounts of H_2) as promising hatcheries for life have made the Wood–Ljungdahl pathway appear as the most promising candidate for the ancestor of all extant bioenergetic chains. This notion is bolstered by phylogenetic results on several enzymes involved in the pathway and most notably the molybdo/tungstopterin proteins

performing the first step of reducing CO_2 to the formyl-moiety and the [NiFe] hydrogenases furnishing reducing equivalents. The almost "inorganic" aspect of the catalytic sites of other enzymes in the pathway, such as CODH or [NiFe] hydrogenases, is also in line with great ancestry of this mechanism [136,137]. Intriguingly, all methanogens found so far are Euryarchaea, and acetogens have up to now only been detected and studied in Bacteria. This dichotomy has led to the proposal that the very divergence of Archaea and Bacteria might actually have been driven by the bifurcation towards either methane or acetate as final product of CO_2 reduction [118,138,139]. In this model, the LUCA would have represented a pre-divergence state using a mixture of both pathways [139].

The "more evolved", higher ΔE_m chains, such as shown in Fig. 2, involving liposoluble H_2 carriers and multiple coupling sites would then have come only later, *i.e.* after the Archaea/Bacteria divergence. The beauty of this model lies in its simplicity and internal coherence and it thereby has attracted considerable attention over the past 10 years. However, a few nagging problems need to be resolved before we can consider this model to represent the final words on the origin of bioenergetics:

Molecular phylogenies indicate the presence of several enzymes performing redox conversions of quinones in the LUCA. Prominent examples comprise the Group 1 [NiFe]-hydrogenases and their membrane-integral cytochrome *b* subunits [50,112], the Rieske/cyt*b* complexes [133,140], quinol-oxidising NO-reductases [78,141–143], polysulfide reductase [70] and formate dehydrogenase [70]. As we have argued before [92,93], the species distribution of the various chemical types of quinones suggests that menaquinones may have been a component of pre-LUCA bioenergetic chains (Fig. 4). Of course, the ability to synthesise menaquinone and thus to run higher ΔE_m electron transfer chains, may have been transferred laterally. This hypothesis was recently tested by reconstructing the phylogeny of genes involved in quinone and methanophenazine biosynthesis (Ducluzeau and Basset, unpublished results). The observed pronounced Archaea/Bacteria split for key gene sequences of the dominant menaquinone biosynthesis pathway instead further supports the likelihood of the presence of menaquinone in the LUCA.

We are aware that all these phylogenies could also be rationalised by very early lateral gene transfers, *i.e.* after the Archaea/Bacteria diversification but prior to the radiation of each domain. Still, we feel that the ever-increasing number of predicted pre-LUCA, quinone-involving enzymes is alarming and requires consideration. These results clearly are at odds with a scenario envisaging minimal Wood–Ljungdahl pathways in the LUCA and a subsequent bifurcation into a methano- and an acetogenic version in Archaea and Bacteria, respectively.

Caution also seems required against putting too much emphasis on the abovementioned simplicity and low-energy arguments. Equating simplicity with antiquity appears to us to lack irrefutable logical consequence as already mentioned before. The energy argument that low ΔE_m bioenergetic chains likely precede the high ΔE_m systems sounds plausible but is actually at odds with the 2nd law of thermodynamics. High ΔE_m (and hence high ΔH) redox couples have a better chance to lower ΔS and thus generate structure and ordered flow of metabolites. Small but finite amounts of redox substrates more oxidising than CO_2, such as Fe^{3+} and/or nitrogen oxides and oxyanions (see above) are likely to have been present even on the early Earth and might impose amendments to the standard model so far exclusively based on the Wood–Ljungdahl pathway. We feel that the comparative study of extant bioenergetic processes as exemplified by the articles in this issue has the potential of providing more solid bases for inferences on energy metabolism at the origin of life.

Acknowledgements

The authors would like to thank David Kramer (East Lansing/USA), Nick Lane (London/UK) and Bill Martin (Düsseldorf/Germany) for numerous stimulating discussions and helpful comments.

MJR's contribution to this publication was carried out at the Jet Propulsion Laboratory, California Institute of Technology, under a contract with the National Aeronautics and Space Administration: with joint support by the NASA Exobiology and Evolutionary Biology award (NNH06ZDA001N) and NASA Astrobiology, Science and Technology Exploration Program (ASTEP) as well as that from the NASA Astrobiology Institute (NAI-Icy Worlds).

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