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Review

Free energy conversion in the LUCA: Quo vadis?☆

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ABSTRACT

Living entities are unimaginable without means to harvest free energy from the environment, that is, without bioenergetics. The quest to understand the bioenergetic ways of early life therefore is one of the crucial elements to understand the emergence of life on our planet. Over the last few years, several mutually exclusive scenarios for primordial bioenergetics have been put forward, all of which are based on some sort of empirical observation, a remarkable step forward from the previous, essentially untestable, *ab initio* models. We here try to present and compare these scenarios while at the same time discuss their respective empirical weaknesses. The goal of this article is to harness crucial new expertise from the entire field by stimulating a larger part of the bioenergetics community to become involved in "origin-of-energy-metabolism" research. This article is part of a Special Issue entitled: 18th European Bioenergetic Conference.

1. Introduction

1.1. Chemiosmosis' comeback to centre stage

Almost 50 years ago, a proton concentration gradient across the mitochondrial inner membrane was finally recognized to mediate between redox disequilibria resulting in electron flow and the chemical disequilibrium of the ADP/ATP couple: the chemiosmotic coupling was discovered [1]. Chemiosmotic coupling was quickly identified as the free energy-transducing principle not only in mitochondria but also in chloroplasts and photosynthetic purple bacteria [2–6]. In the following decades, these three systems were intensely investigated leading to a detailed understanding of the thermodynamic and (somewhat later) of the structural underpinnings of the chemiosmotic mechanism in general [7–10]. Maybe because of the strongly biophysical and in particular thermodynamic aspects of this mechanism, the mainstream biologists increasingly became disenchanted with chemiosmosis proper and turned to regulation, disease-, ageing- and apoptosis- as well as biomass-related topics of the respective free energy harvesting systems.

Widely unnoticed by this mainstream bioenergetic community, microbiologists over the last 20 years have prepared the ground for a renewed interest in the functional principles of chemiosmosis. Chemiosmotic, *i.e.* ion-gradient mediated, ATP production mechanisms turned up in virtually every newly discovered phylum of the prokaryotic world [11]. The employed redox substrates and corresponding electron transfer chains building up the chemiosmotic membrane potential, however, were found to differ wildly from the traditional systems [12].

The concomitant advent of small subunit rRNA phylogeny of prokaryotes [13] for the first time permitted the grounding of research into the evolutionary roots of life on this planet on empirical data rather than on plausibility and *ab initio* inferences. As has already been noticed by Schrödinger in 1944 [14] free-energy conversion or, in biological terms bioenergetics, necessarily had to serve as a midwife to life's origin as well as being a fundamental mechanism of its subsequent evolution.

Despite the widespread occurrence of chemiosmosis, the tacit assumption that the simple but energy-inefficient fermentation and not chemiosmosis was life's primordial way to keep ATP levels highly persistently unchallenged until not so long ago [15]. Two reasons may explain biologists' clinging to the fermentation-first paradigm: (a) The nascent field of microbial bioenergetics seemed to suggest that the examined systems represented a disparate and heterogeneous collection of more or less unrelated mechanisms. (b) The fermentation-first hypothesis represented one of the major pillars of the then (and to some extent still) sacrosanct "primordial-soup" hypothesis for the origin of life [15].

Advances in all fields of microbial bioenergetics (*e.g.* [16,17]) have in the last 20 years definitively done away with the notion of disparate and unrelated chemiosmotic systems. The previously astonishing ubiquity of the fundamental principle, *i.e.* the proton (and in a few cases sodium cation) gradient as the coupling agent, found its explanation in the emerging molecular unity of the free energy converting electron transfer chains generating this proton gradient. The confusing multiplicity of seemingly disparate bioenergetic systems turned out to be the result of the plugging together of a small number of basic enzymes which

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themselves are built from a construction kit of an even more restricted number of basic protein subunits [18].

We feel that it is fair to say that our present knowledge of phylogeny of involved enzymes and their distribution in extant species strongly favours chemiosmosis over fermentation as life's primordial way to harvest free energy (for more detailed arguments, see [15]). Furthermore, as mentioned above, fundamental thermodynamics require free energy conversion to be a crucial prerequisite for life to emerge [19], and a better understanding of the evolutionary history of the chemiosmotic principle will almost certainly contribute to constraining the number of origin-of-life scenarios by confronting predictions from hypothetical models with empirical evidence gathered from extant life. The effort to understand this evolutionary history is on its way to becoming an important research discipline in bioenergetics. This discipline attempts to arrive at an in-depth understanding of the detailed function and the thermodynamic parameters of the diverse free energy harvesting systems of prokaryotes and thus brings hard-core chemiosmosis back into the spotlight of the general field of bioenergetics.

1.2. Reconstructing chemiosmosis' evolutionary past

Contemporary biology's standard method to infer family relationships (phylogeny) within protein families relies on (protein or gene) sequence comparisons [20]. Chemiosmotic electron transfer chains are made up from inorganic and organic cofactors some of which (*e.g.* quinones, NAD(P)) participate directly as integral entities in these chains whereas others (such as iron sulphur clusters, haems or flavins) are fixed in very specific locations of protein-based scaffolds yielding the cofactor-containing enzymes and enzyme complexes. Whereas these proteins are directly amenable to phylogenetic analyses through comparisons of their primary sequences, the "free" cofactors seemingly escape such approaches. However, many of them require biosynthetic enzymes for their synthesis the evolutionary history of which may be inferred *via* phylogenetic analyses.

The most ancestral bioenergetic system we can infer using empirical results is that of the Last Universal Common Ancestor, LUCA, widely considered as a consortium of protocells indulging in extensive exchange of genes and which eventually gave rise to the prokaryotic domains of the Archaea and the Bacteria. As detailed before [21], several criteria can be put forward to judge whether the phylogeny of a given gene suggests its presence in the LUCA. First of all, the respective gene (or protein) needs to be present both in Archaea and Bacteria. The phylogeny of the gene/protein sequences should fall into separate archaeal and bacterial subtrees with a few selected cross-domain representatives not necessarily being an obstacle since horizontal gene transfer certainly does play a role in evolution. The root of the tree, if it can be determined (see below), needs to be positioned in between the archaeal and the bacterial subtrees. The topologies of the individual archaeal and bacterial subtrees should furthermore be roughly in line with species trees based on small subunit rRNA.

Several hurdles appear when trying to use the phylogenetic method for deducing ancestral chemiosmotic pathways. A fundamental limitation lies in the extreme evolutionary timescale of certainly exceeding 3 billion years which separate extant biological systems from earliest life. In order to allow a sufficient phylogenetic resolution, the number of informative sites (*i.e.* bases or amino acids the mutation rates of which are not restricted by functional or structural constraints) must exceed a certain threshold. In our experience, proteins with less than 40 kDa frequently yield unreliable phylogenetic trees. As described in detail previously [22,23], injecting structural and functional information into multiple sequence alignments can sometimes improve confidence levels of specific trees.

However, even for sufficiently long sequences, a principle obstacle hampering straightforward deduction of ancestry from individual protein/gene phylogenies stems from the fact that tree topologies only reflect different degrees of sequence similarities but do not *per se* yield the temporal sequence of divergences. Additional information is therefore required to infer which branching node is the most ancestral one in a given tree and thus to assign a temporal sequences of divergences eventually leading to the observable extant species. In molecular phylogeny, this information is usually obtained by including a sequence or sequences from genes or proteins which clearly share a common evolutionary origin but which have diverged prior to the appearance of the studied protein family. Thanks to their modular make-up, most bioenergetic enzymes lend themselves to such "rooting" approaches since they often share specific protein subunits with other bioenergetic or even non-bioenergetic enzymes [18]. To cite just one example, a root for the Rieske/cyt*b* complex tree has been inferred based on the composite phylogeny of the Rieske subunit present both in this complex and in the functionally unrelated enzyme arsenite oxidase [23,24].

Just as if these fundamental problems of tree reconstruction weren't enough, the procedure is furthermore prone to numerous data glitches. Confounding paralogs with orthologs, sequencing errors (especially inducing frameshifts) or, most simply, failure to recognise a given gene in the genome, will hamper drawing the correct conclusion from reconstructed phylogenies. The case of the recently discovered, low-branching member of the green sulphur bacterial phylum, *Ignavibacterium* (*I.*) *album*, nicely illustrates the problem of gene identification. The non-photosynthetic *I. album* was reported to be devoid of a Rieske/cyt*b* complex [25] and it was therefore concluded that the phylum of the green sulphur bacteria had laterally imported the genes for this enzyme only shortly before the radiation of the truly photosynthetic representatives [25]. A recent comprehensive survey of Rieske/cyt*b* genes from sequenced genomes [26] also doesn't feature *I. album* as carrying these genes. Now, the genes encoding the Rieske/cyt*b* complex are clearly present at loci IALB_1666 and IALB_1667 and they are arranged in the canonical order of a well-behaved Rieske/cyt*b* complex. The more recently sequenced close relative of *I. album*, *Melioribacter roseus* also contains these genes (at loci MROS_1171 and 1772). So why is it so difficult to recognise those genes? The reason lies in the fact that the gene encoding cytochrome *b* is fused to that coding for a hydrophilic multihaem *c*-type cytochrome which in fact accounts for almost 2/3 of the whole sequence length. Simple BLAST searches therefore fail to pick up sufficient similarities between the *I. album* cytochrome *b* gene and standard query sequences from Rieske/cyt*b* complexes. Only a comprehensive phylogeny of all these sequences can decide whether lateral gene transfer plays a role in the green sulphur bacterial phylum (which we think it does; to be published elsewhere) while the previous conclusions [25] based on the erroneous notion of absence of these genes were premature.

In addition to these methodological intricacies, there is the problem of completeness of the dataset. We probably have at best a few percent of the microbiological diversity on our planet available in the form of protein or gene sequences. More than once, we have seen phylogenetic trees "evolve" in front of our eyes while adding newly sequenced representatives of an enzyme superfamily. Worse, the vast majority of prokaryotic phyla are likely to never have made it to the present day but have gone extinct sometime along the over 3 billion years since the days of the LUCA.

The method of phylogenetic tree reconstruction is thus sentenced to forever remain imprisoned in the realm of probabilities. While this method is the most powerful tool at our hands, at the same time it more often than not leaves plenty of room for disagreement and controversy. Nevertheless, to add a grain of optimism, we would hold that basic thermodynamic principles (after all, bioenergetics is all about thermodynamics, isn't it?) can frequently be invoked to prefer one phylogenetic scenario over another (we have illustrated this approach, for example, in our discussion on the evolution of the enzyme arsenite oxidase [27]).

Despite all these caveats, the paramount importance of bioenergetics' evolutionary history for understanding life's origin and its subsequent unfolding over the aeons commands that we do our best to advance our

comprehension of this research topic and that we constructively work towards resolving apparent disagreements. Several mutually exclusive scenarios for the earliest bioenergetics have sprung up during recent years but we feel that the specific premises and corollaries of these models are not always clear to the general field of bioenergetics. In the following we therefore try and describe where these scenarios differ and which empirical results they are based on. We hope to thus initiate further research (and ideally attract newcomers to participate) in this field to eventually reduce discrepancies and ambiguities and advance the frontiers of our understanding of the bioenergetic bases for life's origin.

2. Wood–Ljungdahl *versus* quinone-based ancestral bioenergetics

Whereas the notion that energy harvesting in the LUCA was chemiosmotic, *i.e.* based on an ion-concentration gradient as the coupling agent between redox reactions and ATP/ADP disequilibria, gained substantial acceptance during recent years, the mode of ion-gradient build-up is a hotly debated topic. Martin and Russell [28] proposed a scenario which considers the LUCA to rely on a free energy converting system closely related to the so-called Wood–Ljungdahl pathways operating in extant homoacetogenic Bacteria and methanogenic Archaea. This pathway relies exclusively on redox reactions occurring in the aqueous internal compartment (the "cytoplasm") of the LUCA [29]. A single transmembrane ion pump couples one of these redox reactions to translocation of a proton or, more frequently, of a sodium ion. The generated ion gradient is then harvested by rotor/stator-type ATP synthases (see Fig. 1A). Probably the most intriguing aspect of this scenario lies in the fact that it only requires the redox substrates H_2 (as electron donor) and CO_2 (as the acceptor) [30]. Both these gases are likely to have been strongly abundant in specific environments at the times of the LUCA [31]. It is argued that divergence of this ancestral pathway towards pure acetogenesis gave birth to the Bacteria whereas the Archaea are considered to derive from LUCA's second daughter who chose to rely entirely on methanogenesis [28]. Whereas this scenario is supported by a great number of elegant and intriguing arguments with respect to palaeogeochemistry, it fares less well when it comes to phylogeny. Acetogens and methanogens of the type considered in this scenario are rare on the phylogenetic tree of prokaryotes and furthermore do not occupy the lowest branches of this tree (see [12]). However, as argued recently [32], the so-called phylogenomic argument which stipulates that rare traits on the tree of species are unlikely to be ancient, disregards the fact that the "energy-substrate environment" of the present Earth is certainly very different from that of our planet more than 3 billion years ago. Therefore, truly ancient bioenergetic mechanisms are bound to persist only in restricted anaerobic niches of our extant environment and thus necessarily will tend to be rare on the phylogenetic tree of prokaryotes. Extant organisms from the lowest-branching phyla of the tree may also have lost these pathways. A phylogenetic analysis of the enzymes involved in acetogenesis and methanogenesis, however, indicates that only a few of them fulfil the mentioned criteria for being pre-LUCA [32], calling for further studies on these pathways.

A quite different scenario emerged during the last decades from phylogenetic analyses of several key enzymes involved in very diverse bioenergetic chains. Phylogenetic trees of the Rieske/cyt*b* complexes rooted by the outgroup Rieske subunit from arsenite oxidases were interpreted to indicate the presence of the enzyme in the LUCA [23,24]. The sole function of the Rieske/cyt*b* complexes, however, is to optimally generate a proton gradient from the oxidation of quinol in

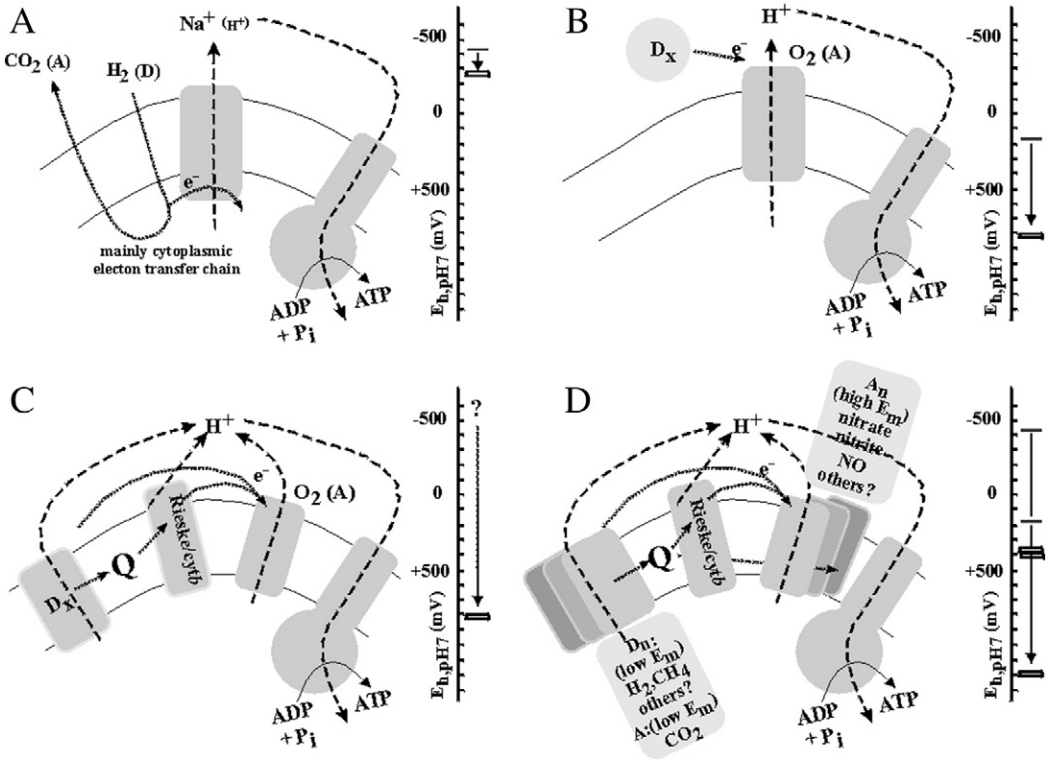

Fig. 1. Schematic outline of the major conflicting scenarios for bioenergetic free energy conversion in the LUCA as discussed in the text as well as their electrochemical characteristics. A corresponds to the Wood–Ljungdahl-type scenario discussed for example in [52]. (D) and (A) stand for donors and acceptors, respectively, and their respective electrochemical potentials are indicated on the right side redox scale as filled (donors) and open (acceptors) bars. Redox midpoint potentials are indicated with respect to the Standard Hydrogen Electrode (SHE). B, C and D represent scenarios considering the availability of high potential acceptors but differing by the exact chemical nature of this oxidant which, respectively, was O_2 in B, C or likely nitrogen oxides and oxyanions as in D. As an example for a donor substrate directly reducing the O_2 reductase in B we chose arsenite ($E_m = +60$ mV) due to the existence of extant bacteria using this pathway [27]. Scenarios C, D posit the additional operation of quinone-based electron transfer chains in the LUCA. The multiplicity of electron donors and acceptors indicated in D (for candidates see [58]) does not necessarily mean that all those processes must have occurred simultaneously in one specific cellular entity since in all scenarios the LUCA is envisaged as a consortium of cells that exchange genetic information and metabolites in a relatively promiscuous manner.

the Q-cycle mechanism. The presence of Rieske/cyt*b* complexes in the LUCA would therefore inexorably call for the co-existence of (some sort of) quinone. In addition, since the redox potentials of all quinones known to operate in biology are substantially higher than the potentials of the CO_2/acetate or the CO_2/methane couples, *i.e.* the oxidants of the Wood–Ljungdahl pathways, a pre-LUCA Rieske/cyt*b* complex furthermore entails the necessity for a terminal electron acceptor substantially more oxidising than CO_2.

2.1. A strong oxidant in LUCA inferred from O_2/NO-reductases; one phylogeny, two solutions

In the vast majority of extant prokaryotes, Rieske/cyt*b* complexes provide electrons for aerobic respiration of O_2 or anaerobic respiration of nitrogen oxides and oxyanions. The haem/copper O_2 reductases of aerobic respiration and the NO reductases of the anaerobic reduction of nitrogen compounds belong to the same superfamily which suggests the composite phylogeny of these two enzymes as an ideal tool to tackle the question of the putative primordial oxidant.

Phylogenetic trees of this superfamily have been grown in several labs over the past 2 decades [33–38,24]. Reassuringly from the methodological perspective, the general topologies of all these trees look the same. However, the robustness of this topology was vexing to all the labs involved in this approach since the tree stubbornly resisted standard interpretation attempts. Rather than suggesting a straightforward conclusion, the topology of the superfamily in fact sends contradictory messages. Two different approaches were taken to overcome the apparent inconsistencies of this tree. Brochier-Armanet and collaborators [36] concluded that the mutual rooting of the individual subfamilies must be unreliable and went ahead to analyse each subfamily separately while strongly relying on the abovementioned phylogenomic argument that only enzyme families with good representations in most phyla are eligible for being pre-LUCA. These authors conclude on a pre-LUCA presence of the so-called A- (or SoxM-) subfamily and infer that O_2 was likely the primordial oxidant (Fig. 1B, C). Since widespread O_2 is considered highly unlikely by the majority of the palaeogeochemical community, LUCA must in this scenario have thrived in unique niches enriched in O_2 by some ill-defined mechanism [39,40]. As a consequence of the stipulated ancestry of the A/SoxM-subfamily, which in fact consists of the low O_2-affinity enzymes adapted to high O_2-tensions, the emergences of the high-affinity groups B/SoxB and C/*cbb*$_3$ are proposed to reflect later adaptations towards microaerophilic niches [41].

We have in the past chosen to take the observed topology of the composite tree at face value and have rationalised the apparent inconsistencies by stipulating that O_2 reductases arose several (at least two) times independently from ancestral NO reductases [24]. This rationalisation was guided by the observation that an amino acid residue that was crucial for O_2- but not for NO-reduction was found in completely different positions on the protein sequence [42]. Ironically, this interpretation suggests exactly the opposite of the previous scenario, that is, pre-LUCA presence of NO reductase (and, by inference, of the respiration of nitrogen oxides and oxyanions, see Fig. 1D) but the late emergence of aerobic respiration [24] as well as the emergence of low-affinity O_2-reductases from within the high-affinity group as O_2-tensions started to rise (ms in preparation).

We are confident that the discovery of new members of the group as well as the biochemical characterisation of the less-studied clades will help to eventually arrive at a consensus with respect to the evolution of the superfamily.

Irrespective of the choice of scenario rationalising the confusing messages of the composite O_2/NO reductase tree, the phylogeny of the superfamily indicates that one of its members was probably present in LUCA and therefore argues for "some" strong oxidant available in the environment of the LUCA, in line with the conclusions drawn from the Rieske/cyt*b* tree. As discussed recently, if strong oxidants were available to the LUCA, a novel scenario invoking methanotrophy as the ancestral way of harvesting free energy becomes conceivable [32].

As mentioned above, palaeogeochemical data are in general interpreted to argue against the presence of significant O_2-levels in the early Archaean. Wouldn't these data at the same time render NO as oxidant unlikely? In this context we feel it necessary to correct a few misconceptions with respect to the scenario of nitrogen oxides as primordial oxidants. Martin and co-workers [43] have recently argued that substantial concentrations of "high potential electron acceptors such as NO or NO_3^- with midpoint potentials near or exceeding O_2" … "would have shifted the oxidation state of such an environment dramatically". As pointed out by Ducluzeau et al. [24] and elaborated in more detail by van Lis et al. [21] the environmental oxidant would in fact have been nitrate and nitrite. NO certainly is the penultimate source of these nitrogen oxyanions. It is formed by high energy reactions of N_2 and CO_2 in the atmosphere but then rapidly converted to nitrate and nitrite after dissolving into the aqueous phase. Further reduction of the 2-electron redox compounds nitrate and nitrite is kinetically hindered in the absence of suitable catalysts able to mediate between the abundant 1-electron donor Fe^{2+} and these 2-electron compounds. This may have allowed nitrate and nitrite to accumulate in the earliest oceans to the levels required for serving as bioenergetic oxidising substrates. The standard redox midpoint potential at pH 7 of nitrate and nitrite is in the vicinity of +400 mV, *i.e.* a very far shot from the potential of O_2 even at nanomolar concentrations (+700 to +750 mV). The NO generated by the enzymatic reduction of nitrite is further reduced by the abovementioned enzyme NO reductase to N_2O and it is the NO/N_2O couple that features a standard redox midpoint potential above +1 V, that is, strongly exceeding that of O_2. However, NO exists only transiently and attains in extant denitrifyers not more than nanomolar steady-state concentrations [44]. Phylogenetic results [21] indicate that the N_2O to N_2 reduction did not operate in the LUCA (as it still doesn't in many extant denitrifyers) and the resulting effective midpoint potential of the nitrogen oxy-compounds would therefore not deviate noticeably from the roughly +400 mV of nitrate and nitrite. This potential would be sufficient to pull electrons through a Rieske/cyt*b* complex (and thus to build up a chemiosmotic gradient) but would not generate the large scale geochemical signals commonly used to infer the advent of O_2 in the environment. The scenario of a nitrate- and nitrite-rich primordial ocean was in fact first put forward based on palaeogeochemical reasoning [45].

2.2. What difference a Q makes!

Since substrates (*e.g.* arsenite [27]) donating electrons directly to the A- (SoxM-) type O_2 reductase are in principle conceivable, the pre-LUCA presence of this enzyme still leaves room for a "single-pump" chain reminiscent of the Wood–Ljungdahl scenario, albeit operating at substantially different redox potentials (Fig. 1B). The presence of at least a partial denitrification chain and, in particular, of a Rieske/cyt*b* complex in the LUCA, however, would call for the presence of quinone-based, membrane-integral electron transfer chains (Fig. 1C, D), a notion fully alien to the abovementioned Wood–Ljungdahl-type models. It is noteworthy that further bioenergetic enzymes catalysing redox reactions of quinones (such as the [NiFe]-hydrogenases [46–49]) or several molybdo/tungstopterin complexes [22] were reported to have phylogenies indicating their pre-LUCA presence. As cautioned above, all these trees only represent likelihoods and moreover are vulnerable to a plethora of data-related problems. The fact, however, that the possibility of quinone-mediated electron transfer in the LUCA is suggested independently by the phylogeny of several unrelated enzymes makes us think that this option cannot easily be wiped off the table.

So what are the "cons" arguing against the Q-mediated chains? We feel that the strongest argument presently put forward is based on present knowledge of the biosynthetic pathways of quinones and haems [43]. Not only are the bioenergetic systems shown in Fig. 1C, D

based on quinone-mediated, membrane-crossing electron transport but most involved enzymes also use haems as essential cofactors. Both in Bacteria and in Archaea, these haems generally are haem *b*, *i.e.* Fe-protoporphyrin IX, although in some species other porphyrin-derivatives may occur [50]. The haem *b* biosynthesis pathway studied in *Escherichia coli* has for a long time been taken to be the one and only pathway employed by prokaryotes. The ground-breaking work of Bali et al. [51] however, has shown that a second pathway for the production of haem *b* and using sirohaem rather than protoporphyrinogen IX as precursor is present in Archaea and δ-proteobacteria. Lane and Martin [52] have interpreted this result as demonstrating that Archaea and Bacteria have invented haem *b* biosynthesis independently and that therefore LUCA must have been devoid of haem *b*. This interpretation was subsequently tested by Sousa et al. [43] by performing a genomic survey of the presence of the respective biosynthesis pathways in major phyla of Archaea and Bacteria. According to Sousa et al. [43] the genes involved in the *E. coli*-type protoporphyrinogen IX pathway are present in all major phyla of the Bacteria (apart from the δ-proteobacteria) whereas Archaea generally feature sirohaem-pathway genes. Sousa et al. therefore conclude that haem *b* biosynthesis is absent from the LUCA. Although this work is undeniably pursuing a promising approach for settling this question, we cannot help noticing that the respective article only presents summarised results but not the relevant details. However, looking at selected details, it is not clear to us how the global results were arrived at. To give just one example, the low-branching phylum of the actinobacteria is classified by Sousa et al. [43] as unambiguously falling into the *E. coli* group of biosynthetic enzymes. However, a BLAST search for the gene coding for the terminal enzyme of the *E. coli* pathway, HemH, which serves to insert the iron atom into the protoporphyrin IX moiety, shows that only 10 members of the 711 sequenced actinobacteria contain this gene. The clear absence of this gene in *Corynebacterium glutamicum* is particularly disturbing to us since we have seen haem proteins from this species not only as genes in the genome but also in optical and EPR spectra recorded on membranes and purified enzymes from this organism. Similar problems exist for several other genes of the *E. coli* pathway in actinobacteria. The lack of a detailed description of the genes considered by Sousa et al. [43] unfortunately prevents us from finding out where we might have gone wrong in our quick testing of their results.

We do think that, considering the crucial importance of this question, an in-depth phylogenetic analysis of all enzymes involved in both the sirohaem and the protoporphyrinogen IX pathways is required. It is obvious that if the conclusions of the Sousa et al. [43] article hold up in such an analysis, the scenarios of Fig. 1B, C, D will be facing trouble.

Whereas such a result may not yet spell the end of these scenarios since one might imagine simpler porphyrin molecules functioning in the respective enzymes in the LUCA, a second argument put forward by Lane and Martin [52], if borne out, would indeed be devastating to the models of Fig. 1C, D. This argument refers to the biosynthesis pathways of quinones. In the same vein as for haem biosynthesis pathways, Sousa et al. [43] interpret the results of their genomic survey of menaquinone-biosynthesis genes as indicating the presence of two distinct pathways in Archaea and Bacteria and thus the absence of menaquinones (and hence quinones in general) in the LUCA. In contrast to the case of haem biosynthesis where so far we have only sporadically tested the results presented in the Sousa et al. article as discussed above, one of us has in the recent past analysed the very same pathways and arrives at the opposite conclusion (A.-L. Ducluzeau, unpublished). The respective results suggest a substantial overestimation in the study by Sousa et al. [43] of the men-gene dependent pathways in Thaumarchaea, Thermoproteales, Thermoplasmatales and the Deinococcus/Thermus groups, most probably due to homolog/ortholog attribution problems. Apart from these data-related discrepancies, the inclusion of phylogenetic analyses of the considered genes in addition to mere distribution among species yields a picture significantly different from that painted by Sousa et al. [43].

Sousa et al. [43] furthermore constitute a list of core-genes likely to have been present in the LUCA in order to infer "what sorts of functions might have been present … in the common ancestor…". For joining this list, a gene is required to be present "in at least one member each of 20 out of 30 major eubacterial taxonomic groups … and present in at least one member each of all 11 major archaebacterial taxonomic groups sampled…". This criterion may seem innocent at first sight but it certainly isn't. The crucial part in there is the "of all 11 major archaebacterial taxonomic groups". The Archaea contain phyla which are fully devoid of quinones (the quinone-free methanogens, see discussion in [12]) and consequently also lack quinone-redox-converting enzymes. However, in recent phylogenies of the Archaea, these phyla aren't low-branching and may therefore be derived —having lost quinone-biosynthesis and quinone-based energy metabolism at some point in their evolutionary history. The criterion of having to be present in ALL 11 major archaeal taxa by definition excludes quinone-based chains from ever being considered. The efforts by Sousa et al. [43] in setting up their list are laudable but without looking at a single genome, the outcome was clear from the start since it was built into the selection criterion. It's not the data that disqualify quinone-based bioenergetics, it's the way to exclude unpleasant data that disqualify this approach.

Of course, it can be argued that abiotically produced precursor molecules of haems and quinones might have played the respective roles in the LUCA. We refute this emergency exit as profoundly "anti-Occam'ish". It is difficult to see why these precursors would have been exchanged independently by the same biosynthetically derived cofactors both in Archaea and in Bacteria. To our knowledge, no facile abiotic synthesis pathways for porphyrins or naphthoquinone moieties have been described so far. By contrast, all evidences converge towards a picture of the LUCA as a biosynthetically elaborate entity with a nucleotide-coded metabolism.

In summary, we consider that a thorough analysis of the genes coding for the biosynthesis enzymes producing haem *b* and menaquinones will provide crucial information to assess the viability of the models stipulating quinone-based, membrane-spanning bioenergetic electron transfer chains in the LUCA.

3. The question of the ancestral ion pump

Based on molecular phylogenies of rotor/stator-type ATP synthases, Mulkidjanian and collaborators have proposed that the ancestral ion gradient was in fact based on sodium ions rather than on protons [53]. This proposal was initially grounded in the observation that sodium-driven ATP synthases are found dispersed over many phyla of both Bacteria and Archaea and that they therefore must derive from an ancient trait present in the LUCA. Incidentally, we would like to remark that the identical argument works equally well (or even better, given their extraordinary predominance) to favour ancestry of H^+-dependent ATP synthases. More recently, a composite tree including an outgroup enzyme was interpreted to support the Na^+-early scenario [54]. A sodium gradient as the coupling agent in the LUCA would predict that the redox reactions building up the ion gradient might also pump Na^+ rather than H^+. Quinones, of course, can only shuttle H^+ but not Na^+ across the membrane and the Na^+-early scenario is therefore also in conflict with the scenarios of Fig. 1C and D. Moreover, as neither O_2- nor NO-reductases can pump sodium ions, a pre-LUCA presence of these enzymes as shown in Fig. 1B, C, D and discussed above would not jive with a sodium-based ion gradient in the LUCA.

Since the coupling enzymes of acetogenesis and methanogenesis in general pump Na^+, there is an obvious convergence of interests between the proponents of the Wood–Ljungdahl scenarios and the Na^+-early hypothesis. Lane and Martin [52] have consequently adopted the Na^+-early idea and have integrated it into their scenario to yield an elegant solution to the problem of the transition of the pre-LUCA metabolic reactions fuelled by a pre-existing H^+ gradient in hydrothermal vents and the active ion-pumping of the LUCA and its descendants.

The H^+/Na^+ question has therefore become central to the debate on the likely bioenergetic principles operating in the LUCA. We fully acknowledge this problem and consider that a thorough understanding of the nature of the ancestral ion gradient will allow a considerable reduction of the number of viable scenarios.

We have therefore analysed the published phylogenies of both rotor/stator-type ATP synthases and H^+-translocating pyrophosphatases (for which an ancestral Na^+ translocation mechanism was also stipulated [55,56]) with great interest and scrutiny. We have to admit that it remains unclear to us how the respective authors arrived at the conclusion of ancestral Na^+ gradients based on these trees. Neither the trees of rotor/stator-type ATP synthases nor those of H^+/Na^+-translocating pyrophosphatases fulfil the pre-LUCA criteria outlined above with respect to their Na^+-dependent members. Since we certainly may be biased, we invite the interested readers to investigate the respective articles for themselves.

We would like to reiterate that we do not say that the Na^+ scenario is necessarily wrong. However, a detailed review of the published data leads us to the conclusion that the evidence is much less convincing than is expressed in the respective articles. In the same vein, we do not affirm that the phylogenies leading to the scenarios of Fig. 1B, C, D are famous last words.

In this context, it is noteworthy that the evolutionary history of the Rieske/cyt*b* complexes has recently been reinterpreted to fit the quinone-free model [26]. The new scenario stipulates that the ancestral Rieske/cyt*b* complexes actually are the b_6f-type enzymes and that the enzyme was distributed over the prokaryotes after the Archaea/Bacteria divergence *via* lateral gene transfer. As in the case of the haem/copper-oxidase superfamily mentioned above, the topology of the tree presented by Dibrova et al. [26] strongly resembles those of previous trees [23,57]. The difference in scenarios thus does not arise from novel data but from diverging interpretations with respect to the arrow of time on the tree defining ascendance through the nodes. Or to put it more prosaically: it depends on which clade is interpreted as the most ancient. Let us mention only two caveats: (1) Positing the b_6f-related clades as the most ancestral ones is in flagrant conflict with the rooting of the phylogeny by outgroup sequences as mentioned above [23,24]. (2) The argument for ancestral b_6f complexes is based on the detailed topology of the respective b_6f-containing clades [26]. The majority of the corresponding nodes feature bootstrap values below 20% (descending as low as 8%). A bootstrap value of 20 means that only 20% of the trees reconstructed in bootstrap juggling contain this node whereas 80% feature different branching topologies! Similarly low bootstrap values in the same region of the tree have been observed previously [57] but were taken to preclude specific inferences concerning this part of the trees. The advent of further key sequences and/or of relevant 3D structures allowing structure-guided sequence alignments may help to improve reliability of the concerned branching orders.

We can't help noticing that the species which are presently discussed as performing Na^+-dependent chemiosmosis live at the minimal thermodynamic edge of redox substrate driving forces (see the discussion in [12]). If this intriguing correlation should hold up in future surveys, it may be worthwhile looking into thermodynamic constraints rather than evolutionary history to rationalise sodium-based chemiosmosis in the respective species.

4. Afterword

Commensurably with the involved geological timescales and the diversity of habitats, the evolutionary history of bioenergetics is a multifaceted research topic. Debates and controversies exist in many areas of this field just as in other fields. Most of these controversies, however, pertain to particular types of metabolisms or specific evolutionary transitions during Earth's history. The question on the origin of bioenergetics and, by simple extrapolation, on the origin of life occupies an outstanding position in the field of the evolution of bioenergetics. The furthest we can probably go back in time using empirical results is the bioenergetic repertoire of the LUCA. Intriguingly, a number of conflicting scenarios for LUCA's bioenergetic outfit have been put forward during recent years emphasising the growing interest in this question. We therefore considered it useful to outline not only where the field currently stands, what the competing models look like and what they are based on, but also what we consider untenable arguments or ambiguous databases. As discussed in this article, there are a number of obvious approaches that potentially will provide decisive evidence. In addition, however, there certainly are innumerable further approaches able to shine light on these questions and it is the major goal of this article to raise the interest of a larger part of the bioenergetics community and stimulate them to get involved in this research.

Note added in proof

A reassessment of the proton-pumping capacities of A-, B- and C-type O_2 reductases advocates an evolutionary ancestry of B- and C-type enzymes over the low affinity A-type one [V. Rauhamaki & M. Wikstrom, The causes of reduced proton-pumping efficiency in type B and C respiratory heme-copper oxidases, and in some mutated variants of type A, BBA Bioenergetics, this issue.

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