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# On the antiquity of metalloenzymes and their substrates in bioenergetics☆

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ABSTRACT

Many metalloenzymes that inject and extract reducing equivalents at the beginning and the end of electron transport chains involved in chemiosmosis are suggested, through phylogenetic analysis, to have been present in the Last Universal Common Ancestor (LUCA). Their active centres are affine with the structures of minerals presumed to contribute to precipitate membranes produced on the mixing of hydrothermal solutions with the Hadean Ocean ~4 billion years ago. These mineral precipitates consist of transition element sulphides and oxides such as nickelian mackinawite ($[Fe > Ni]_2S_2$), a nickel-bearing greigite (~$FeSS[Fe_3NiS_4]SSFe$), violarite (~$NiSS[Fe_2Ni_2S_4]SSNi$), a molybdenum bearing complex (~$Mo^{IV/VI}{}_2Fe_3S^{0/2-}{}_9$) and green rust or fougerite (~$[Fe^{II}Fe^{III}(OH)_4]^+[OH]^-$). They may be respectively compared with the active centres of Ni–Fe hydrogenase, carbon monoxide dehydrogenase (CODH), acetyl coenzyme-A synthase (ACS), the complex iron–sulphur molybdoenzyme (CISM) superfamily and methane monooxygenase (MMO). With the look of good catalysts – a suggestion that gathers some support from prebiotic hydrothermal experimentation – and sequestered by short peptides, they could be thought of as the original building blocks of proto-enzyme active centres. This convergence of the makeup of the LUCA-metalloenzymes with mineral structure and composition of hydrothermal precipitates adds credence to the alkaline hydrothermal (chemiosmotic) theory for the emergence of life, specifically to the possibility that the first metabolic pathway – the acetyl CoA pathway – was initially driven from either end, reductively from $CO_2$ to CO and oxidatively *and* reductively from $CH_4$ through to a methane thiol group, the two entities assembled with the help of a further thiol on a violarite cluster sequestered by peptides. By contrast, the organic coenzymes were entirely a product of the first metabolic pathways. This article is part of a Special Issue entitled: Metals in Bioenergetics and Biomimetics Systems.

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*It is the inorganic elements that bring organic chemistry to life* David Garner

## 1. Introduction

It is a commonplace that Nature benefits from the catalytic properties of transition metals such as Fe, Ni, Cu, Zn, Co, Mo, W, V and Mn which she uses copiously in various kinds of metal enzymes [1–4]. Indeed, a vast number of exergonic but kinetically hindered (due to high activation barriers) chemical reactions are quickened by the presence of metal catalysts and many industrial processes take advantage of the same phenomenon [5]. Less considered is the role *via* specific $2e^-$ electrochemical properties of metals such as molybdenum and tungsten in free-energy converting enzymatic machines that closely couple these exergonic reactions to the endergonic ones; the assailing of which is what life at bottom is all about [6–8]. However, the question arises in the consideration of the emergence of life and biological complexity as to whether the utilization of metal ions as catalytic entities was a founding requirement [9–11], or if metal ion-based catalysis represents an evolutionary improvement over pre-existing enzymes which performed the same reactions and energy conversions, albeit more sluggishly, with organic rather than metal cofactors [12]. Several of the authors of this contribution have in the past addressed the evolutionary pedigree of enzyme systems involved in biological free-energy conversion, an approach developed further here [7,9,13–24].

The picture emerging from our phylogenetic investigations has made us feel uneasy with the idea of metal-free enzymes pre-dating later metal-containing ones, at least with regard to bioenergetic systems. One might argue that bioenergetic systems are bad examples since their task is to perform electron transfer reactions and thereby they are intrinsically prone to recruit redox active metal centres. On

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the other hand free-energy converting mechanisms must have been among the earliest, or even THE earliest, along the metabolic pathways of nascent life [7,9,10,13–16,20,21]. Life is an out-of-equilibrium state of Nature and constitutes an enormous decrease in entropy of the thermodynamic space that it thrives in within the cell whilst exporting entropic waste to the surrounds. Life therefore requires free-energy-(disequilibria-) harvesting capabilities as its most fundamental prerequisites [6–8,11,18,19,25]. We consider it likely that bioenergetic enzymes are among the oldest biocatalytic systems, and thus we need to understand the temporal sequence of cofactor usage in bioenergetic systems. Indeed, perhaps the most striking feature of the submarine alkaline hydrothermal model for the emergence of life that we adopt here [10,26] as a context for our investigation is the remarkable extent to which the essential elements of pre-life's first days are to be found intact, according to this model, as "living fossils" and their "casts", throughout all three domains of extant life: iron (nickel) sulphide clusters in ferredoxins [27,28], hydrogenase and CODH/ACS [7,10,16,29–34], variable valence iron in di-iron methane monooxygenase [35], molybdenum or tungsten atoms in pterins [7,20,36,37], and how some of these centres are ensconced in peptide nests [38–40].

In this work we have collected relevant information on the evolutionary history of bioenergetic systems available in the literature. This analysis is confined to those enzymes that convert the penultimate bioenergetic substrates, that is, the redox couples injecting and extracting reducing equivalents at the beginning and the end of these electron transport chains, respectively, whilst neglecting intermediate electron transfer complexes which couple redox reactions to the build-up of chemiosmotic potential (such as the Rieske/cyt*b* complexes) and the purely electron shuttling "doxins" (*e.g.*, ferredoxins, cupredoxins, and flavodoxins). We also disregard post-LUCA metalloenzymes such as nitrogenase and the Oxygen Evolving Complex that, notwithstanding their affinities with mineral structures, likely were biochemical fabrications from the start as judged from their intricate biological assembly pathways. As transpiring from the preceding points, we consider only chemiosmotic free-energy conversion for this analysis, leaving aside substrate-level, fermentative pathways. This does not significantly narrow the scope of the compilation since the chemiosmotic processes dwarf fermentative ones with respect to diversity, efficiency and hence importance to life [41,42]. Moreover, fermentative pathways appear to be relatively young innovations of life on Earth and, in many microbes, are only brought to bear when deprived of electron acceptors and, thereby, chemiosmotic oxidative phosphorylation [41,43–45].

Chemiosmotic energy conversion is fuelled by electrochemical disequilibria induced by the simultaneous presence in the environment of reducing and oxidizing substrates. Microbiological screening has, during the last two decades, unraveled an unanticipated variety of redox substrates in microbes. The range of electron donors and acceptors exploited by life vastly exceeds the obvious choices such as hydrogen, methane or hydrogen sulphide as reductants and oxygen, sulphate and nitrogen oxides as oxidants and go as far as the seemingly unlikely substances selenate [46], arsenics [22,47–50], phosphite [51] or even xenobiotics such as ethylbenzene [52] or chlorate/perchlorate [53]. Whilst this craving of prokaryotic life for any possible kind of redox substrate may appear stunning, it makes perfect sense from the thermodynamic perspective since it is only the secure maintenance of disequilibria that keeps life away from the unforgiving vortex of its entropic demise imposed by the 2nd law of thermodynamics [6,8,25].

## 2. "Early" and "late" substrates for chemiosmotic energy conversion

Admittedly, the multitude of chemiosmotic electron transfer chains is still far from being comprehensively understood on the molecular level [4]; even less so when it comes to phylogeny. Nevertheless, several enzymes converting redox substrates have now been studied by phylogenetic approaches although the compilation of cases we show in Fig. 1 may have omitted representatives escaping our attention.

One of the crucial parameters pertinent to emergence-of-life scenarios is the question of the nature of the primordial substrates, whether autotrophic (basically inorganic) or heterotrophic (small organic molecules). Fig. 1 is split into halves. The left side features autotrophic substrates, whereas the right half contains information on enzymes dealing with heterotrophic substrates. Both halves are subdivided into columns containing bioenergetic electron donors *and* acceptors, respectively, whilst the vertical axis indicates the redox midpoint potentials of the various redox couples with respect to the standard hydrogen electrode (SHE). Enzymes likely to have been present in the Last Universal Common Ancestor (LUCA) by virtue of their molecular phylogeny are shape-coded by ellipses whereas rectangles denote those that seem to have emerged more recently. The entity LUCA stands for life giving rise to the divergence of the prokaryotic domains Archaea and Bacteria which is estimated to have occurred more than 3 billion years ago, and maybe long before that. It represents the form of life closest to its origins that can be assessed by molecular phylogenetic approaches. A given enzyme is identified as potentially pre-LUCA if its phylogenetic tree shows a clear cut divergence into archaeal and bacterial subtrees (occasional cross-domain lateral gene transfers notwithstanding [24]), if furthermore the tree topology by and large corresponds to current species trees and if, for the cases where rooting is possible, the root falls in between the Archaea and the Bacteria. For details on the respective phylogenies we refer the reader to the corresponding articles listed in the Fig. 1 legend.

Strikingly, we failed to find any pre-LUCA enzymes involved in terminal electron transfer reactions associated with heterotrophic metabolisms. By contrast, all but one of the inorganic electron donating substrates were oxidized by pre-LUCA enzymes. Concerning inorganic electron acceptors, $CO_2$ and sulphur (polysulphide) appear to be pre-LUCA whereas most higher-potential acceptors are reduced by post-LUCA enzymes. This makes geochemical sense since under the anaerobic conditions of the early Earth these inorganic substances almost certainly were present in their reduced states and thus unavailable as electron acceptors. Notable exceptions are the three nitrogen oxides and oxyanions, *i.e.* nitrate, nitrite and nitric oxide, which have been argued, based on molecular phylogenies, to be reduced by pre-LUCA enzymes [15,17]. Again, palaeogeochemical conditions suggest large-scale abiotic sources for these nitrogen oxides and oxyanions in the Hadean and early Archaean as discussed previously [15,63] (Fig. 2). The presence of these strong oxidants during the ages of the LUCA, although predicted by palaeogeochemists more than two decades ago [64] is far from being generally accepted by the geochemical community. Given the paramount importance of the question of availability of strong oxidants for scenarios on the earliest metabolism [7], further empirical results both from geochemistry and from biology are badly needed.

One surprise in all this is the lack of evidence for the use of $H_2S$ as an electron donor in the LUCA. This may follow from the dearth of hydrogen sulphide in the earliest ocean due to buffering by iron–nickel sulphide equilibria in magma-driven hydrothermal systems and the stripping of sulphide from high temperature solutions prior to exhalation of which more later [65–67].

The pattern seen in Fig. 1, which groups organisms by physiology based on fundamental redox couples involved in respective metabolisms, thus suggests pre-LUCA life to have exclusively thrived on autotrophic substrates (Fig. 3). This also argues against heterotrophic organic soup type models for the origin of life and favours the autotrophic scenarios, such as the pyrite-pulled surface metabolism [68] and the alkaline hydrothermal (chemiosmotic) vent model described in the next section (Fig. 2) [7,8,69].

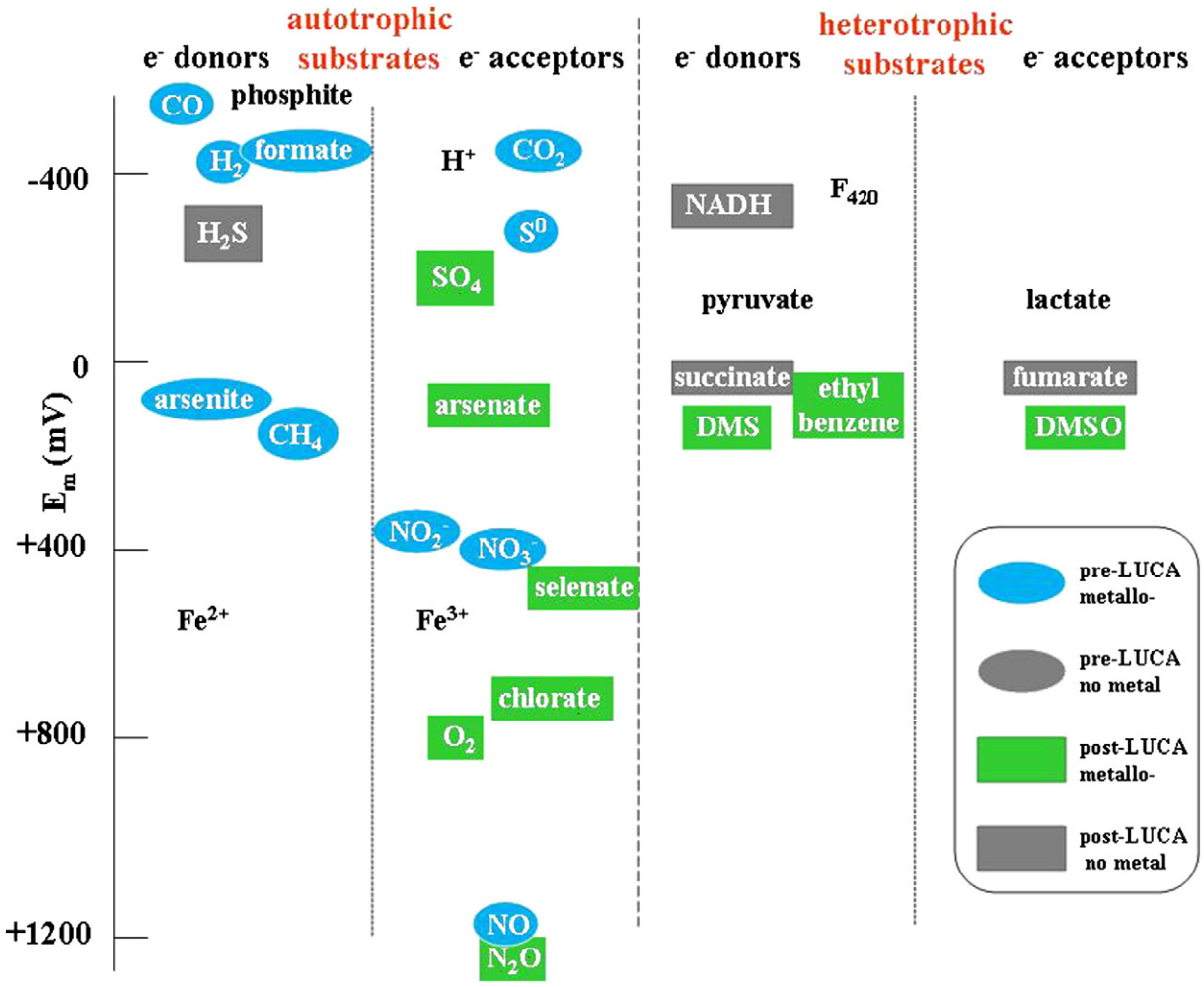


**Fig. 1.** Proposed utilization of bioenergetic redox substrates (inorganic/autotrophic or organic/heterotrophic) already in LUCA or only after the Bacteria/Archaea divergence as indicated by phylogenetic results. Pre- and post-LUCA characteristics as well as metal content are as indicated in the lower right scheme of the Figure. The following phylogenetic studies underlie this figure: [11] ($H_2$), [54] ($CO_2$, CO), [20] (formate, $CO_2$, $S^0$, DMSO), [55] ($SO_4$), [47] (arsenite), [22] (arsenate), [56] ($H_2S$), [57,58] (selenate, chlorate, DMS, and ethylbenzene), [59–61] (succinate dehydrogenase, fumarate reductase), [17,62] ($NO_3^-$, $NO_2^-$, $N_2O$, NO, $O_2$). The substrates not associated with ellipses or rectangles appear relevant to the topic of this article but we were unable to find phylogenetic data allowing conclusions of their pre- or post-LUCA nature.

## 3. The submarine alkaline hydrothermal (chemiosmotic) model for the onset of metabolism

In its most recent formulation the chemiosmotic model holds that hydrothermal hydrogen was one source of electrons, atmospheric carbon dioxide dissolved in ocean water was one source of carbon and that hydrothermal methane was a source of both electrons and carbon to emergent metabolism ~4.4 billion years ago [7,8] (Fig. 2). The two fluids met and interacted to produce a porous inorganic precipitate mound which acted as catalytic flow reactor and trap, frustrating the immediate intermixing of alkaline with acidulous fluids [8,10]. Judging from the modern hydrothermal counterpart, Lost City, the convective system operating in serpentinizing ocean crust could have fed aqueous fluids of pH 10 to 11 and around 100 °C to the primeval ocean of pH 5.5 to 6 over a period of at least 100,000 years (or, assuming a more appropriate time scale, for $>10^{17}$ μs) [74]. Hydrogen concentrations would have exceeded 15 mmol/l and methane, more than 2 mmol/l [75–77]. Minor concentrations of ammonia and hydrogen bisulphide and trace concentrations of molybdenum were likely also fed to the hydrothermal mound in the alkaline fluids [78–80]. On its part the somewhat acidic ocean bearing carbon dioxide and nitric oxides would also have supplied ferrous and lesser ferric iron (as oxyhydroxide [81]) as well as other minor transition metals to the mound through entrainment in the hydrothermal updraft. Calcium concentrations in the hydrothermal fluid were likely higher than ambient ocean water, sodium concentrations would have been the same and magnesium and silica probably less than concentrations in the then ocean [77,82].

It would be a mistake to think that the Hadean Ocean remained in a stable state for long periods. After all, we are trying to imagine the world one third of the age of the Universe ago. Meteorite impacts of various masses and at various velocities would have bombarded what was almost certainly a water world, cosmic and solar dust may have blocked solar radiation on some occasions; cosmic and UV radiation and gamma-ray bursts from hypernova may have been very much stronger then, and ocean temperatures and acidities could have oscillated about extremes. Though mainly comprising carbon dioxide with lesser nitrogen oxides and various sulphur species, atmospheric pressures too could have fluctuated perhaps from one to at least ten times the present [83]. Yet in the ocean depths the alkaline hydrothermal effluent, born of serpentinization, was both well buffered (at pH 10 to 11) and thermostated (at ~100 °C) [8,69]. Depending on conditions and depth we imagine the hydrothermal mound to comprise ephemeral carbonates, amorphous silica, white and green rust (fougerite) and mixed transition element sulphides dominated by iron but including nickel, cobalt, zinc and molybdenum and/or tungsten [3,70,73,84–87]. The hydrogen and methane remained in solution as the main potential electron donors at concentrations around 15 and 2 or more millimoles per litre, respectively [75,88,89] (Fig. 2).

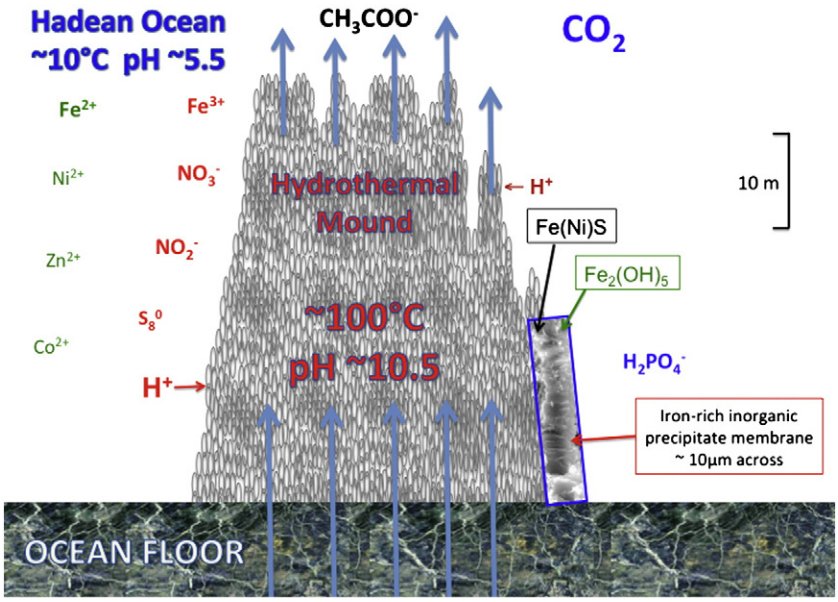


**Fig. 2.** Sketch of hypothetical submarine porous hydrothermal mound composed of silica, oxides, sulphides and ephemeral carbonate argued to be life's hatchery [8,69]. The blue arrows represent the flow of the alkaline hydrothermal solutions. The inorganic membranes involved in metabolism (blue framed inset), measuring ten or so nanometers across, would have had an exterior of ferrous hydroxide (white rust, $Fe(OH)_2$) – a precipitate prone to oxidize to green rust ($\sim[Fe^{II}Fe^{III}(OH)_4]^+[OH]^-$) – whilst the inner zones of this outermost membrane comprised nickelian mackinawite (Fe(Ni)S), a mineral that transforms to greigite ($\sim FeSS[Fe_3NiS_4]SSFe)_n$) at ~70 °C [70,71]. The sulphide is also likely to be dosed with molybdenum as clusters approximating $Mo^{IV/VI}{}_2Fe_3S^{0/2-}{}_9$ [72,73].

## 4. Metals as the venerably oldest catalysts

The fact that the transition metals could have constituted much of the spontaneously precipitated inorganic barriers separating the acidulous ocean from the alkaline hydrothermal fluid is in concert with the distribution of redox enzymes containing metal cofactors as compared to those relying on organic cofactors for catalysis [3,4]. In Fig. 1 metalloenzymes are coloured whereas enzymes operating with organic cofactors are grey. All pre-LUCA enzymes found to be associated with the generation of chemiosmotic potential included in this analysis are

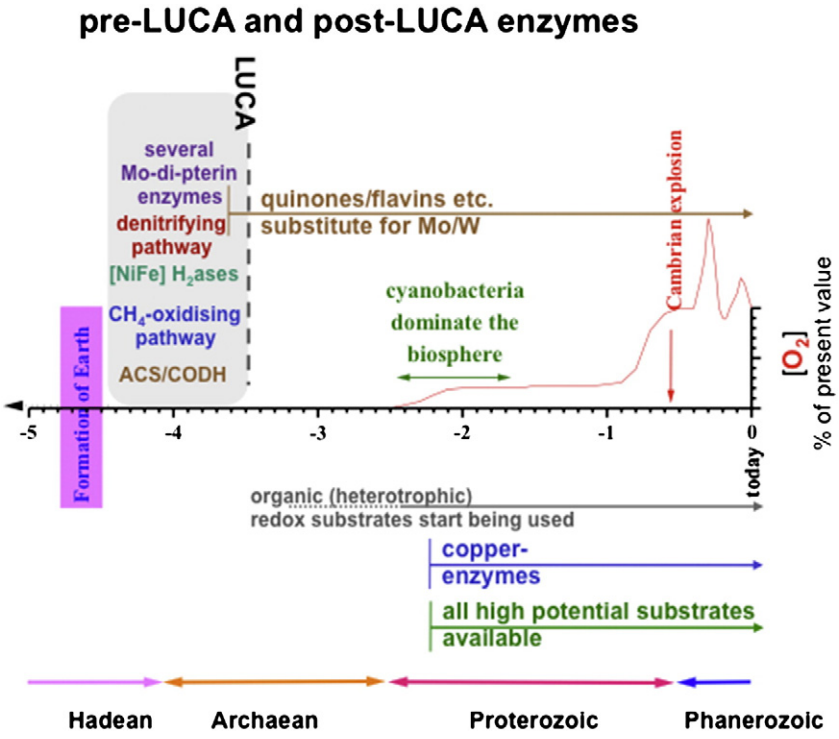


**Fig. 3.** Schematic timeline of the emergence of bioenergetic substrates and of the dedicated enzymes from life's origin through the ages of LUCA to the oxygenated times of our present planet as indicated by phylogenetic arguments. The time region of the LUCA as well as that of the actual origin of life are rough estimates whereas the onset of significant atmospheric $O_2$ is well-constrained by palaeogeochemical evidence.

metalloenzymes, many of them ligated to inorganic sulphide atoms. We did not find a single pre-LUCA bioenergetic enzyme which runs solely on organic cofactors. By contrast, several post-LUCA enzymes use these organic cofactors and they predominantly convert heterotrophic substrates, underscoring the conclusion drawn above concerning the ancestry of autotrophic over heterotrophic energy metabolism.

The picture emerging from Figs. 1 through 3 therefore is one of LUCA drawing its energy from inorganic redox couples and exclusively using metalloproteins to this end. Bioenergetic redox conversions of substrates using organic cofactors appear to have been co-opted by life only after the Bacteria/Archaea divergence. As will be detailed below, organic cofactors in general, such as quinones or flavins, appear to have been added to the bioenergetic inventory during the development of the LUCA, likely driven by life's striving to overcome concentration limitations of some of the metal catalysts.

These findings turn the catalysis-improving-metal-cofactors point of view on its head. Metalloenzymes seem to be older than enzymes not reliant upon metals, at least in bioenergetics. As we have seen, this makes sense too from a geochemical point of view in that there would have been no dearth of Fe, Ni, Co, Zn and W in the Hadean Ocean ultimately derived from the magma-driven very hot acidic springs exhaling into the acidulous Hadean Ocean [1–3,65,87,89–91]. And the submarine alkaline hydrothermal springs would have supplied molybdenum and tungsten at ~100 nmol/l [18,79,92–95].

The autotrophic scenarios for the origin of life of course predict such a scheme of events. More specifically, the alkaline hydrothermal vent (chemiosmotic) model stipulates metabolism to have started exclusively based on metal catalysts such as iron, nickel, cobalt, molybdenum, and tungsten [10,14,16,18,95] though peptides would soon be synthesized in the alkaline hydrothermal mound and stabilize and begin to optimize metal-based catalysis as well as to introduce substrate-specificity [40]. The pattern of phylogenetic ancestry of bioenergetic enzymes thus bears witness of the “rocky roots” of energy metabolism [7,14]. We turn now to those potentially catalytic metal sulphides with clear affinities with the structures of the active centres of the metalloenzymes.

## 5. Metal sulphides and oxyhydroxides as pre-enzymatic catalysts

Iron is the commonest transition metal on Earth. It was also the commonest transition metal in early oceans in the form of $Fe^{++}$, derived from magmatically driven hot acidic springs at ~400 °C [61,65,79,87]. It precipitated in the hydrothermal mounds as iron sulphides on meeting submarine alkaline hydrothermal springs of moderate temperature (≤100 °C) which provided at least a portion of the sulphide as $HS^-$ [29,70,78,95]. The main form of the precipitate was in the semiconducting iron monosulphide mackinawite [10,70,96–99]. This sulphide, that may also contain a few percent nickel replacing the iron in so-called nickelian mackinawite, is built of $Fe_2S_2$ or $FeNiS_2$ rhombs (Fig. 4) [97,100,101]. At temperatures of around 70 °C some of the mackinawite oxidizes to the inverse thiospinel, greigite ($\sim FeSS[Fe_4S_4]SSFe)_n$) [70]. Greigite comprises a 4Fe4S cubane in which the four iron atoms are octahedrally coordinated, balanced by two tetrahedrally coordinated iron atoms. Greigite too can contain nickel, where it probably substitutes for an octahedral iron in the cubane structure [100–105] (Fig. 4). Were nickel to be locally concentrated, then the discrete thiospinel, violarite, is precipitated ($\sim NiSS[Fe_2Ni_2S_4]SSNi)_n$) (Fig. 4) [95,105].

Nickel is well known to be an effective hydrogenation catalyst in industry [5] and Huber and Wächtershäuser [106] have shown it to be effective also in the assembly of methane thioacetate from carbon monoxide and methane thiol — a reaction comparable to the enzymatic role of acetyl coenzyme-A synthase (ACS). In this regard we note the structural affinity between the active centre of ACS and that of the minerals greigite and violarite which suggests the possibility of a such a catalytic role for these minerals or their fine clusters (Fig. 4). By the application of density functional theory (DFT) calculations and Monte Carlo simulations, Haider et al. [105] demonstrate the relative immiscibility of solid state greigite and violarite. Whether this immiscibility holds at the cluster level, particularly in the presence of other ligands, is still an open question. However, accepting these findings [105] we are not only drawn to compare the violarite structure to ACS, but also the greigite structure's affinity to carbon monoxide dehydrogenase (CODH) [79] (Fig. 4).

Amino acids, the building blocks of peptides, have been synthesized in conditions comparable to those obtaining at Hadean submarine alkaline hydrothermal vents by Huber and Wächtershäuser [107] through the amination of carboxylic acids. Again using nickel–iron catalysts these same authors have also demonstrated the polymerization of amino acids to short peptides in similar context [108,109]. These experimental findings, involving iron and iron–nickel sulphides as catalysts, sit comfortably in the alkaline hydrothermal mound scenario for the generation of organic molecules to be expected in the hatchery of life [10]. And the fact that ferrous hydroxide appears equally effective as catalyst in the amination of carboxylic acids [107] adds a certain redundancy to minerals comprising the hydrothermal mound. This is because iron hydroxides comprise a portion of the inorganic membrane as synthesized under comparable conditions in the lab [70,110]. We examine the catalytic propensity of the iron hydroxides and oxyhydroxides next.

Ions of iron by themselves are both positively charged and highly oxidizing. For $Fe^{+++} \Longleftrightarrow Fe^{++}$ $E_m = 0.8$ V — a potential exploited effectively by anaerobic methane oxidizing organisms with the concomitant reduction of green rust (or fougerite $\sim[Fe^{II}Fe^{III}(OH)_4]^+[OH]^-$) at 90 °C [112–115]. However, ferrous hydroxide oxidizes to green rust (or fougerite) without the intervention of life. The oxidation states of fougerite range from $\sim[Fe^{II}{}_3Fe^{III}(OH)_8]^+[Cl.2H_2O]^-$ to $\sim[Fe^{II}Fe^{III}(OH)_4]^+[OH]^-$). In the case where water is the oxidant it is assumed hydrogen is generated [116–123], a second source of this fuel required for life's emergence [8,10,124]. Green rust has one other striking “prebiotic-like” catalytic effect. Nitrate, a likely component of the earliest oceans [15,63,125], is reduced to ammonia concomitantly with oxidation of sulphate-bearing green rust [126–129]. The ammonia could thereby directly aminate carboxylic acids in the same outer zone of an inorganic membrane. Judging from its structural similarity, green rust may have also catalyzed the oxidation of methane. For example, methane monooxygenase which effects such an oxidation in the methanotrophs [35,130,131], consists of two octahedrally coordinated iron atoms – oscillating from $Fe^{II}$ through $Fe^{III}$ to $Fe^{IV}$ and back – with two oxygen bridging ligands. The basic arrangement of the iron and oxygen (and nitrogen) atoms is comparable to the minimal structure of green rust highlighting the interplay between structural catalysts and metabolic substrates in submarine off-axis hydrothermal vent areas, both modern and ancient [[8,35,70,132,133] (Fig. 5).

For all the examples of minerals shown in Figs. 4 and 5 it is their overall resemblance in spatial arrangement of metals to the enzyme cofactors that suggests the latter to have emerged from nanocrysts or clusters of the former. However, if we look for presumable catalytic activities of these minerals in the scenario of the alkaline hydrothermal mound, it is well possible that a major share of the catalytic workload was performed by only a fraction of the crystalline material. Two subpopulations come to mind: (a) surface-exposed clusters which necessarily must somewhat deviate from their bulk counterparts shown in Figs. 4 and 5. Surface areas do have the additional appeal to be easily accessible by bulkier and strongly charged molecules. Of course, electroneutral gases such as $H_2$ or $CO_2$ probably can deeply penetrate the mineral barriers and need not rely on surface chemistry. For these cases, however, a second type of deviation from bulk crystalline order may be important, that is, (b) lattice defects [111]. A comparison of the mineral- and the protein-bound clusters in Fig. 4 indeed suggests that small deviations from the general lattice structure may help to improve catalysis. Whereas the

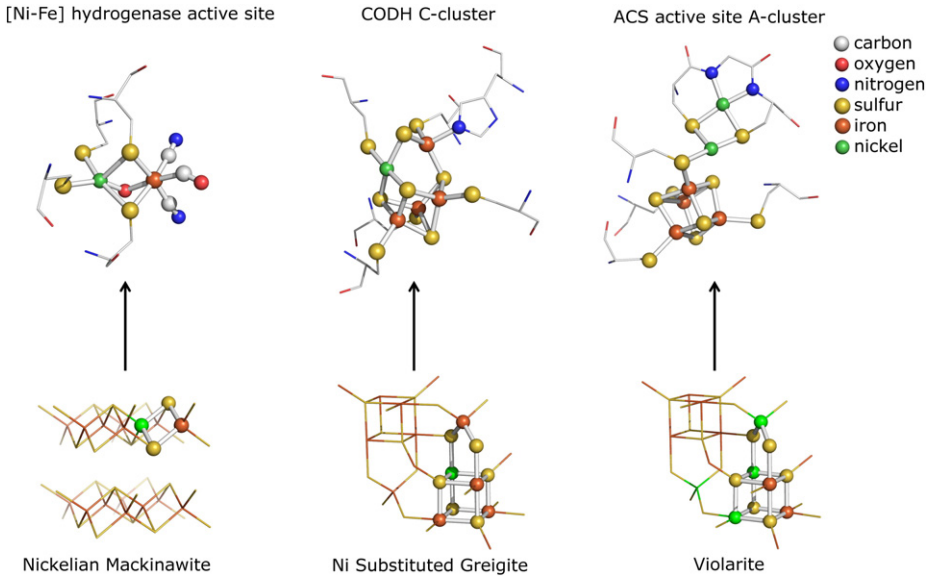


**Fig. 4.** Diagram demonstrating the affinities between the natural sulfide nickelian mackinawite on the bottom left, greigite (*e.g.* (~FeSS[$NiFe_3S_4$]SSFe) centre, and violarite (*e.g.* (~NiSS[$Ni_2Fe_2S_4$]SSNi) on the right [100–102,105] with the active centres of early metalloenzymes above: Ni-Fe hydrogenase, CO dehydrogenase (CODH) and acetyl coenzyme-A synthase (the active site or A cluster, ACS) respectively. Defects in the distorted mineral lattice on the surfaces of amorphous nanoclusters offer the most active sites for catalysis [111] prior to the sequestering of the finest clusters by the alpha chain backbone of uncoded short peptides, *i.e.*, enantiomeric peptides with regularly repeating $\propto_R$ and $\propto_L$ conformations delimited by main-chain dihedral angles of the individual residues [38–40].

global structural similarities between the metal cofactors and the minerals may bear witness to their evolutionary relationship, it may well be that it is the sites with defects that play the crucial catalytic roles [134].

## 6. Interlude on the importance of $2e^-$ redox compounds in bioenergetic electron transfer chains

Extant bioenergetic chains appear to crucially rely on the presence and action of $2e^-$ redox compounds, such as quinones, flavins, NAD(P) or $F_{420}$. Two distinct but equally indispensable functional properties of these redox compounds can be invoked to rationalize their central role in bioenergetics.

### *6.1. Interfacing $1e^-$ and $2e^-$ segments of bioenergetic chains*

Many textbooks describe bioenergetic systems as single reducing equivalents flowing "down" a chain of enzymes towards the ultimate electron sink, the terminal acceptor substrate. Real life bioenergetics, however, involves frequent transitions between $1e^-$ and $2e^-$ reactions. The majority of reducing and oxidizing electrochemical substrates in fact are $2e^-$ compounds which are extremely difficult to redox-convert *via* two consecutive single electron redox steps. Prominent examples are formate, nitrate, succinate, arsenite or simply $CO_2$. Many common redox compounds of bioenergetic chains, such as [FeS] centres, hemes or cupredoxins, by contrast, generally shuttle between only two redox states and therefore are $1e^-$ centres. This fact entails the necessity to interface $1e^-$ and $2e^-$ segments of the respective chains, a task fulfilled by what is commonly called "$2e^-$-gates". Quinones and flavins are well-suited for these purposes since, depending on their electrostatic environment (see below), they can either collect or distribute reducing equivalents one by one or act as $2e^-$ redox couples. In the context of this article it is particularly noteworthy that, in addition to the small organic molecules mentioned above, two metals are frequently found in bioenergetic systems to mediate between $1e^-$ and $2e^-$ transfer reactions, *i.e.* molybdenum and tungsten. In the vast majority of members of the superfamily of molybdo/tungstopterin enzymes, these metals catalyse $2e^-$ conversions of their respective substrates whilst redox-interacting with single electron intramolecular redox centres.

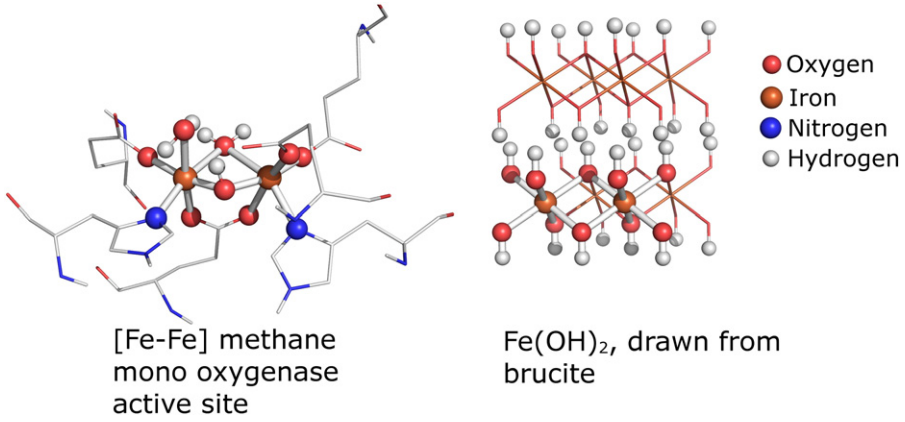


**Fig. 5.** A comparison of the active site of methane monooxygenase with the $Fe_2O_2$ building block of $Fe(OH)_2$, a precipitate highly prone to oxidation to green rust or fougerite ($\sim[Fe^{II}Fe^{III}(OH)_4]^+[OH]^-$) [118,132,133].

### 6.2. Free energy conversion through electron bifurcation

Under certain conditions (see [19]), the above-mentioned compounds are virtually locked in the $2e^-$ redox domain. As discussed in [8,18,19], this property allows the counterintuitive reduction or oxidation of seemingly out-of-range redox centres. The crucial role of these "redox bifurcations" for the mitochondrial respiratory chain has been recognized for more than forty years [135,136] and is increasingly appreciated for many other electrochemically challenging redox reactions [137].

Does this pivotal role of the mainly organic $2e^-$ redox compounds in extant bioenergetics argue against the ancestry of metal catalysts over organic cofactors? It certainly doesn't since in many of the extant systems, molybdenum and tungsten still play exactly these roles [138]. We therefore consider the as yet untested idea that iron sulphide clusters dosed with molybdenum or tungsten, would also have constituted effective "ready-made" prebiotic catalysts. The possibility that molybdenum could have acted in a way similar to that of the molybdopterin cofactors at the very emergence of life is given by the discovery of variable valence molybdenum iron sulphides clusters approximating to $Mo^{IV/VI}{}_2Fe_3S^{0/2-}{}_9$ [72,73]. This kind of cluster, in conjunction with green rust and nickel–iron sulphides, may have catalyzed the onset of a denitrifying methanotrophic acetogenesis as the first metabolic pathway [7]. Since molybdenum and tungsten are present in trace amounts relative to iron, it seems obvious to us that a strong pressure exerted by bioavailability limitations will have forced life early on to come up with "home-made" substitutes for the extraordinary redox wizards molybdenum and tungsten and thus have resulted in the emergence of quinones, flavins and so on.

#### 6.2.1. Early peptides

It is notable that iron, in the form of iron sulphides, is the commonest type of metal in present day proteins. We have also noted that amino acids, the building blocks of peptides, have been synthesized in conditions comparable to those obtaining at Hadean submarine alkaline hydrothermal vents through the amination of carboxylic acids [107]. Peptides are also relatively easy to synthesize in such conditions, in contrast to nucleic acids and phospholipids, [108,109,139–141]. Independent of their side chains, the backbone has a marked distribution of partial charges along its length [38]. Thus the backbone, depending on its conformation, is attracted to appropriate ions through hydrogen bonding, other electrostatic interactions or even covalent interactions. Here, we draw attention to ion binding *via* the NH groups and examine how such combinations seem to precurse those metalloenzymes with the deepest pedigrees.

### 6.3. Metal ions and iron–sulphur centres sequestered by peptides

When metals such as Fe, Cu, Ni and Co are incubated with peptides at high pH values they often react by forming complexes with them as in Fig. 6a [142–145]. Such a pattern is seen also in the A cluster (the active site) of the living enzyme, acetyl coenzyme-A synthase, depicted at the top right of Fig. 4, and in several other well known proteins such as serum albumin and the amyloid precursor protein. They bear a certain resemblance to haem groups and may perhaps [40] have performed similar chemical roles to them during early evolution. It is important to appreciate that in these complexes the metal takes the place of the NH hydrogen atom so they contain covalent metal–nitrogen bonds, completely different from the bonds that bind iron–sulphur centres to peptides discussed next. The occurrence of iron-containing peptides is all the more remarkable since the iron in the oceans has now been converted, due to the oxygen in the atmosphere, to $Fe^{III}$, which of course is highly insoluble and many organisms have to go to great lengths to obtain that metal [146–149].

Turning now to iron sulphur centres in proteins, they typically occur as $Fe(cys)_4$, $Fe_2S_2(cys)_4$, $Fe_2S_2(cys)_2(his)_2$, $Fe_4S_4(cys)_4$ or $Fe_3S_4(cys)_3$, where the amino acid cysteine is acting as $RS^-$ bound covalently to an iron atom [150–154]. The majority of these iron–sulphur centres, except those in high potential iron proteins, are also bound *via* S···NH hydrogen bonds to a curved row of main-chain NH groups of successive residues as shown in Fig. 6b. These characteristic and common features of proteins are used in a number of situations as anion-binding concavities in proteins (for phosphates and carboxylates). The concavity has given rise to the name nest for these features [38]. The anion, in this case the iron–sulphur centre, may be referred to as the egg. Given the existence of simple peptides comprising 8 or so amino acids, we envisage that iron sulphur centres would have been stabilized by nests wrapped around them [39,40,156]. These peptides are unlikely to have included cysteines, so the $RS^-$ iron-binding function would have been carried out by alkyl sulphides produced in the hydrothermal mound. Such chelation might be expected to increase the catalytic propensity of the metal clusters comprising the inorganic membranes and prevent both growth and dissolution of angstrom sized clusters [39,40,138].

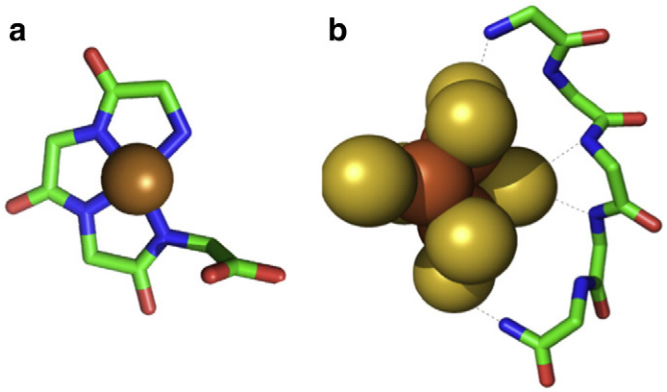


**Fig. 6.** Stick structures of two types of ion-bound polypeptide (side chains omitted). The left hand diagram (a) depicts a nickel-tetraglycine [142,143]. Note the similarity with the protein ligands chelating the distal nickel atom in acetyl coenzyme-A synthase (ACS) as depicted in Fig. 4. The right hand diagram (b) shows a RLRLR [38] nest binding a thiolated $Fe_3S_4$ "egg". This is comparable to the ways peptides commonly enfold such centres in ferredoxin [155]. RLRLR nests are peptides wherein the main chain atoms of successive residues are enantiomeric with conformations alternating between about $-90°$, $0°$ and $+90°$, $0°$, *i.e.*, negative and positive phi angles respectively [38] (carbon, green; oxygen, red; iron, rust; nitrogen blue; sulphur, yellow; nickel, brown).

When iron–sulphur centres assemble, the iron atoms become part of a group that is negatively charged and reducing, or less oxidizing, due to the donation of electrons from sulphur to iron [152]. The frequent binding of iron sulphur centres by main-chain NH groups of peptide nests further modulates the redox potentials of the various iron atoms [153,154].

Fig. 7 indicates the range of possible ionized species of $Fe_4S_4$ and $Fe_2S_2$ centres and their net charges including that of the surrounding cysteine sulphurs. The pairs of species commonly observed in proteins are relatively limited and are shown in red in Fig. 7. The typical pair is that with net charges $2- \Leftrightarrow 3-$ ($E_m = -150$ to $-750$ mV), a notable exception being $Fe_4S_4$ centres in high potential iron proteins, which all lack nests and interconvert the charged forms $1- \Leftrightarrow 2-$ ($E_m = 100$ to 350 mV) [157]. The relevance to peptides is that nests are anion-binding features and Fig. 7 confirms that iron–sulphur centres bound by aliphatic thiols are essentially anionic. Thus in early evolution, with an assumed absence of cysteine residues, short peptides may have formed into nests that bound and stabilized iron–sulphur centres complexed with aliphatic thiols generated in the hydrothermal mound [38–40]. Dongun Kim and coworkers [158] point out that such peptide nests are basically alpha sheets (*i.e.*, repetitive chains of peptide whereby the carbonyls on one side of the chain are all oriented in the same direction and are hydrogen bonded to the amino groups on the neighbouring chain and so on as shown in [40] Fig. 1), an important consideration given

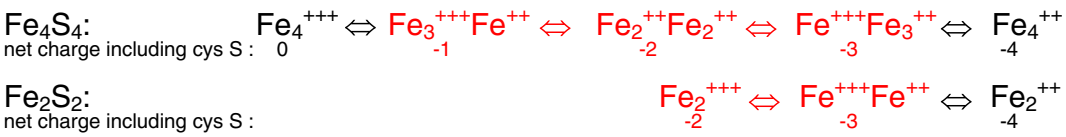


**Fig. 7.** The possible ionized forms of $Fe_4S_4(RS)_4$ and $Fe_2S_2(RS)_4$ iron–sulphur centres. Pairs of species on the left-hand side of the diagram have significantly higher potentials, $E_m$, than the pairs on the right. The net charges include that from cysteine sulphurs as well as that from iron and sulphide atoms. Only certain ionized species, colored red, occur commonly in proteins and for a given centre typically only a couple of interconverting species occur under biological conditions. All centres occurring in proteins have an overall negative charge and those with −2 or −3 are favoured.

that such sheets might have acted as the first organic membranes and retained this function during the organic takeover [39,40,158].

*6.3.1. The organic takeover*

Intriguingly, all bioenergetic enzymes catalyzing reduction/oxidation of inorganic electrochemical substrates for which we found phylogenetic indications with respect to their ancestry, appear to have stuck to their original metal catalysts rather than evolving organic substitutes. This is all the more surprising as some of these metals are likely to have been in short supply. That such a dearth of specific metals did occur is suggested by the fact that a number of bioenergetic roles are nowadays fulfilled by small organic molecules such as quinones and flavins, which were probably even then already present in the LUCA. In the framework of our scenario, these roles must initially have been played by metals, most likely molybdenum or tungsten. Life has thus very early on started to synthesize molecules replacing some of the metal catalysts. It appears likely to us that quinoids and flavins arose to substitute for Mo/W in the high and the low redox potential ranges, respectively. Whereas flavins occur both in membrane-associated and in soluble enzymes, the quinoids appear to have split labour into the liposoluble redox conversions carried out by menaquinones and the hydrosoluble ones performed by pyrroloquinoline quinone (PQQ), thiamine pyrophospate (TPP) and the likes. The flavin moiety in flavodoxin is even able to substitute for the [FeS] protein ferredoxin and one might speculate that the necessity to become parsimonious with iron only arose after the oxidation of the biosphere and the concomitant massive precipitation of ferric iron. The small size of the flavodoxin molecule unfortunately precludes phylogenetic analyses towards deep evolutionary times to test these kinds of speculation.

## 7. Conclusions

When people talk of the building blocks of life they often think of so-called prebiotic molecules said to rain from space or atmosphere to assemble as the makings of the first cells. We take a diametrically opposed view. In our reasoning the building blocks were the metal sulphides and oxyhydroxides that contributed to the walls of the first compartments where the first metabolic pathway of carbon fixation arose, involving reductions and oxidations of C1 entities catalyzed by these same metal-bearing nano-crystalline minerals.

We feel that our view is bolstered by arguments from all four concerned disciplines, *i.e*

(1) Geology: the conditions of the early Earth have the potential to supply all the metals that go to produce the proto-metalloenzymes from acidic ~400 °C springs exhaling into the deep all-enveloping Hadean Ocean, itself somewhat acidic — metals that would precipitate on meeting sulphurous alkaline submarine springs of moderate temperature to comprise the porous mounds that are hypothesized to act as the hatchery of life [10,78].

(2) Physics (thermodynamics): in striking contrast to almost all other origin of life scenarios, the alkaline hydrothermal vent model does not conflict with the 2nd law of thermodynamics [6,41].

(3) Chemistry (catalysis): The active centres of the present day metalloenzymes are so similar to the metal-bearing minerals of the hydrothermal mound as to invite the understanding that they are vestiges of the catalysts co-opted, and later biofabricated, by the very organic molecules they were responsible for producing from scratch — the molecules that primed emergent life [40,70,94,159].

(4) Biology (phylogenetics): The metalloenzymes that inject and extract reducing equivalents at the beginning and the end of electron transport chains involved in chemiosmosis are indicated by their phylogenetic trees to have been present in the Last Universal Common Ancestor (LUCA). The active centres of these very early enzymes are affine with the structures of minerals comprising precipitates produced on the mixing of hydrothermal solutions with the Hadean Ocean >4 billion years ago. These mineral precipitates consist of transition element sulphides and oxides such as nickelian mackinawite ($FeNiS_2$), a nickel-bearing greigite (~$FeSS[Fe_3NiS_4]SSFe$), violarite (~$NiSS[Fe_2Ni_2S_4]SSNi$), a molybdenum bearing complex ($Mo^{IV/VI}{}_2Fe_3S^{0/2-}{}_9$) and green rust or fougerite (~$[Fe^{II}Fe^{III}(OH)_4]^+[OH]^-$). They may be respectively compared with the active centres of Ni–Fe Hydrogenase, carbon monoxide dehydrogenase (CODH), acetyl coenzyme-A synthase (ACS), the complex iron–sulphur molybdoenzyme (CISM) superfamily and methane monooxygenase. With the look of good catalysts – a suggestion that gathers some support from prebiotic hydrothermal experimentation – they could be thought of as the original building blocks of proto-enzyme active centres, when combined with short peptides [9,13,24,39,60,131,138,158].

The convergence of the makeup of the LUCA metalloenzymes discussed in this work with the mineral structures and compositions of hydrothermal precipitates therefore adds further credence to the alkaline hydrothermal (chemiosmotic) theory for the emergence of life, specifically to the possibility that the first metabolic pathway – the acetyl CoA pathway – was initially driven from either end, reductively from $CO_2$ to CO and oxidatively and reductively from $CH_4$ through to a methane thiol group, the two entities assembled with the help of a further thiol on a violarite cluster sequestered by peptides in the precipitate membrane: to wit, life was to emerge from a denitrifying methanotrophic acetogenic pathway [7].

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