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Metal availability and the expanding network of microbial metabolisms in the Archaean eon

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Life is based on energy gained by electron-transfer processes; these processes rely on oxidoreductase enzymes, which often contain transition metals in their structures. The availability of different metals and substrates has changed over the course of Earth's history as a result of secular changes in redox conditions, particularly global oxygenation. New metabolic pathways using different transition metals co-evolved alongside changing redox conditions. Sulfur reduction, sulfate reduction, methanogenesis and anoxygenic photosynthesis appeared between about 3.8 and 3.4 billion years ago. The oxidoreductases responsible for these metabolisms incorporated metals that were readily available in Archaean oceans, chiefly iron and iron–sulfur clusters. Oxygenic photosynthesis appeared between 3.2 and 2.5 billion years ago, as did methane oxidation, nitrogen fixation, nitrification and denitrification. These metabolisms rely on an expanded range of transition metals presumably made available by the build-up of molecular oxygen in soil crusts and marine microbial mats. The appropriation of copper in enzymes before the Great Oxidation Event is particularly important, as copper is key to nitrogen and methane cycling and was later incorporated into numerous aerobic metabolisms. We find that the diversity of metals used in oxidoreductases has increased through time, suggesting that surface redox potential and metal incorporation influenced the evolution of metabolism, biological electron transfer and microbial ecology.

The geosphere and biosphere are linked through the global biogeochemical cycles of major elements and an associated network of electron-transfer reactions[1–3]. Over time, the evolution of these biological reactions has altered the composition of the Earth's atmosphere and oceans[4–7]. Oxidoreductases ('EC1' in the Enzyme Commission numerical classification scheme) are the molecular nanomachines responsible for all biologically driven electron-transfer reactions across the tree of life[8,9]. Oxidoreductase protein families have at least ten different ancient origins, but about half of all metal-containing oxidoreductases share a common ancestor[10]. Horizontal gene transfer (HGT) allows widespread distribution of oxidoreductase genes between distantly related microbes, thus increasing the potential impact of the gene products on biogeochemical cycles[7].

EC1 proteins often contain transition metals[11] such as iron, copper and manganese. Specific metal cofactors are used for different metabolic functions, linking appropriate redox potentials with specific substrates[12,13] (Fig. 1). The availability of different metals and substrates has changed over the course of the Earth's history as a result of changing redox conditions, particularly global oxygenation[8,14]. Changing metal availability led to the expanded utilization of new transition metals, and directly influenced biological innovation and the evolution of new metabolic pathways[2,15–17]. Oxygenation of the atmosphere occurred between 2.4 and 2.3 billion years ago (Ga) and recently has been constrained[18–22] to 2.33 Ga. This Great Oxidation Event (GOE) presumably led to the evolutionary selection of aerobic metabolisms, as supported by bioinformatics analysis of existing genomic data[3,17,23]. Differences in the abundance of Fe-, Zn, Mn- and Co-binding structures suggest that prokaryotes and eukaryotes evolved in anoxic and oxic environments, respectively[17]. These studies demonstrate that metal availability and incorporation had a direct impact on microbial evolution[3,17,23]. Preserved geochemical evidence, such as isotopic fractionation and physical and/or molecular fossils, can be used to date the approximate origin of selected reactions used in central metabolisms. The approximate dates of origin can be compared to the availability of redox metals that were used in oxidoreductases.

In this Review, we examine how the appearance of anoxygenic photosynthesis, methanogenesis, methane oxidation, sulfur reduction, sulfate reduction, oxygenic photosynthesis, nitrogen fixation and nitrification/denitrification is linked to the availability of key metal cofactors in reconstructed global redox conditions. Using extant microbial metabolisms, we further map the co-evolution of metabolic pathways to redox conditions. Our primary goal is to provide a greater understanding of how the geosphere and biosphere co-evolved in the context of the planetary redox state and its influence on the global network of electron transfer. The evolution of other metalloproteins was also influenced by global redox state and metal availability, but our focus in this study is on oxidoreductases and biological electron transfer. An in-depth review of the geochemical proxies that give the best estimates for the dates of emergence of Archaean microbial metabolisms is given in the Supplementary Information and summarized in Supplementary Tables 1 and 2.

Global redox state impact on availability of metal cofactors

Each of the metabolisms mentioned above involves enzymes that use specific transition metals for catalytic function (Fig. 2, Supplementary Table 3). In principle, the origin of these metabolisms can be dated by using preserved isotope fractionations and redox-sensitive metal enrichments in sedimentary rocks, as well as fossil records (Fig. 2, Supplementary Table 2). According to geochemical thermodynamic calculations and sedimentary rock data[19,24–33], the sources and sinks of biologically critical dissolved metals in the ocean have changed through time. The chief inputs

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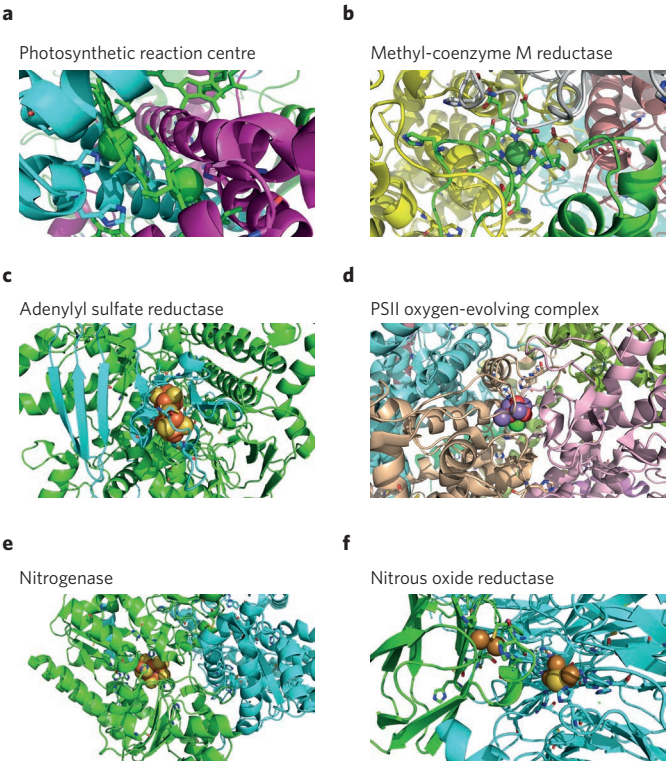

Figure 1 | Protein metallocofactors of core microbial metabolisms. **a**, Photosynthetic reaction centre of purple bacteria, with Mg-bacteriochlorophyll (light green sphere, Mg); Protein Data Bank structure 2BOZ. **b**, Methyl-coenzyme M reductase, with Ni-porphinoid (dark green sphere, Ni), 1MRO. **c**, Adenylyl sulfate reductase, with iron–sulfur clusters Fe_4S_4–Fe_4S_4 (brown spheres, Fe; yellow spheres, S), 3GYX. **d**, Photosystem II (PSII) oxygen-evolving complex, with $CaMn_4O_4$ (green sphere, Ca; purple spheres, Mn; red spheres, O), 1S5L. **e**, Nitrogenase, with $MoFe_7S_8C$ (teal sphere, Mo; brown spheres, Fe; yellow spheres, S; blue sphere, C), 1M1N. **f**, Nitrous oxide reductase, with Cu_4S (light brown spheres, Cu; yellow sphere, S), 1QNI.

include continental weathering processes, aeolian transport and hydrothermal fluids, with the main sink being the formation of insoluble mineral oxides or sulfides.

Sulfur reduction and sulfate reduction are core microbial metabolisms of the Palaeoarchaean era, and potentially the Eoarchaean. The EC1 proteins of sulfur reduction and sulfate reduction require iron, incorporated as FeS clusters, haems and bound ions. Iron was available in the Archaean ocean, and is essential to many other primitive metabolic pathways as well. One such ancient metabolism is methanogenesis, a strictly anaerobic respiratory pathway that uses carbon dioxide as an electron sink. This pathway requires iron, nickel and either molybdenum or tungsten. In principle, molybdenum and tungsten can be exchangeable as transition metals in key enzymes, depending on availability[2,34,35].

The accessibility of iron and nickel in the open Archaean ocean, and of tungsten and molybdenum around hydrothermal vents, not only allowed their incorporation into key enzymes but also fostered fundamentally different mineral environments that selected for biologically energetic requirements[36–38]. Although magnesium is not a transition metal and is not biologically redox-active, as a divalent ion it coordinates porphyrin in virtually all forms of chlorophyll[39]. Magnesium and other elements were supplied to the Archaean oceans by chemical weathering[40–42]. Obviously, anoxygenic photosynthesis would have occurred in the upper ocean, whereas sulfate reduction, sulfur disproportionation/reduction and methanogenesis were present in the ocean interior. All of these metabolisms have arguably ambiguous geochemical signals that entered the geological record between 3.8 and 3.4 Ga (Fig. 2, Supplementary Table 2). Many of the cofactors and proteins among these metabolic pathways share structural motifs such as ferredoxin-like folds (containing different stoichiometries of Fe/S in clusters), all of which were universally appropriated across the tree of life[43].

Genes that evolved during a punctuated period of biological innovation in the early to mid-Archaean eon, which coincides with rapid diversification of bacterial lineages, are likely to be involved in electron-transport and respiratory pathways[23]. Genes arising after this expansion show increased use of redox-sensitive transition metals coinciding with atmospheric oxygenation. The accessibility of negative redox potentials associated with methanogenesis, sulfur reduction, sulfate reduction and anoxygenic photosynthesis (−500 to 0 mV; Fig. 3a)[1,2] in the reducing conditions of the Archaean oceans and the availability of crucial metals resulted in their use. These metabolisms seem to have formed the initial core of the global network of electron-transfer reactions and set the stage for later-evolving metabolisms that would access half-cells with higher redox potential (Fig. 3).

One of the most critical, yet enigmatic, pathways required for life is nitrogen fixation. All life is based on reduced nitrogen (for example, ammonium) in proteins and nucleic acids. Hydrothermal vents and other sources of fixed nitrogen would not have been sufficient to sustain microbial production in the early Archaean before 3.0 Ga (refs 44,45). The three nitrogenase isoforms, containing either iron, vanadium or molybdenum in the active site, have slightly different nitrogen isotope fractionation factors[46,47]. Based on the subtle fractionation differences, there seems to be isotopic geochemical evidence for nitrogen fixation between 3.2 and 2.9 Ga via the Mo-nitrogenase[48]. A primitive form of nitrogen fixation almost certainly evolved earlier than 3.2 Ga (refs 49,50), although isotopic signatures of specific nitrogenase isoforms in older rocks and kerogens are ambiguous, primarily owing to metamorphic alteration[48] (see Supplementary Information).

There was potentially a negative feedback between the evolution of the nitrogen cycle and the oxygenation of the atmosphere and ocean[51]. The evolution of oxygenic photosynthesis, which requires manganese in the Mn_4O_5Ca oxygen-evolving complex of photosystem II (ref. 52), was accompanied by enhanced carbon burial[53] leading to net accumulation of oxygen in the atmosphere. This accumulation of oxygen would have led to biological oxidation of ammonium to nitrite and nitrate (NO_2^- and NO_3^-), which were then potentially lost to the atmosphere through subsequent denitrification[54]. These linked processes can be inferred from positive excursions in the isotopic composition of preserved organic nitrogen[55]. However, once over this barrier, the further oxidation of the oceans at the GOE would have led to the increased availability of molybdenum and copper with concomitant reduction in the solubility of iron and manganese[56].

Copper proteins harness the power of oxygen

Copper is an essential cofactor in enzymes that catalyse the core microbial metabolisms nitrification, denitrification, aerobic oxidation of ammonia, aerobic methane oxidation and aerobic respiration[2] (Figs 2 and 3). The origins of methane oxidation at about 2.9 to 2.7 Ga and nitrification/denitrification at about 2.7 to 2.5 Ga (Fig. 2, Supplementary Table 2) would have required the presence of oxygen to react with methane and ammonia, respectively[55,57]. It is possible that oxidative weathering reactions were present in pockets of oxygen-containing waters produced by oxygenic photosynthesis in biological soil crusts and aquatic microbial mats[58] in the late Archaean before the GOE[59–61]. These oxygen-producing systems could have increased the availability of copper and potentiated early metabolisms involving aerobic oxidation of CH_4 and NH_4^+. Copper isotopic

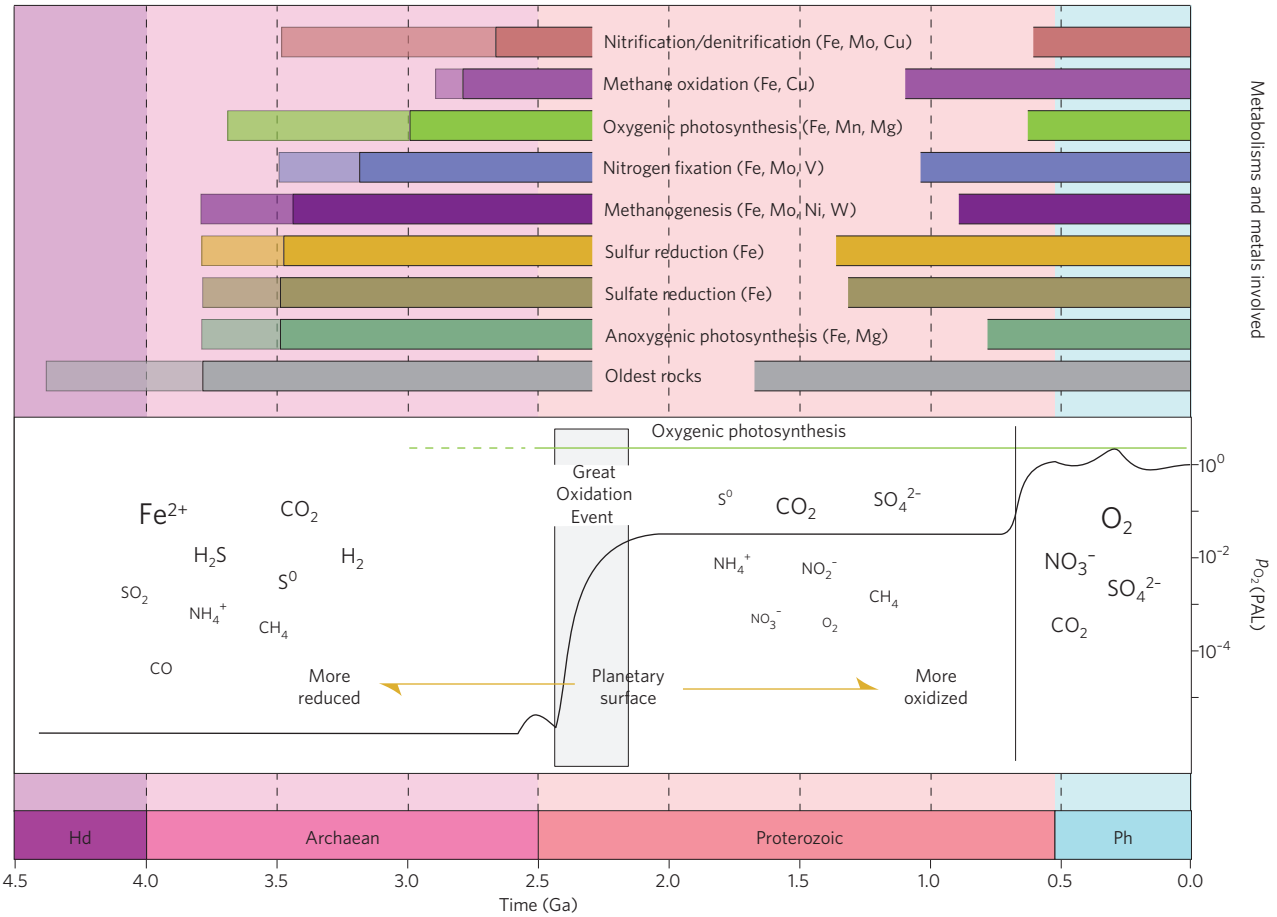

Figure 2 | Preserved geochemical evidence ages of Archaean metabolisms billions of years ago (Ga) and global oxygenation. a, Dark age bars represent more confident ages, and lighter age bars represent less confident ages based on geochemical evidence in Supplementary Table 2. The metals listed in parentheses are used in the oxidoreductase proteins (EC1) associated with each metabolism. **b**, Schematic representation of oxygen's rise throughout Earth history, modified after Jelen *et al.*[2] and Lyons *et al.*[20]. The two main oxygenation events are presented together with the availability of relevant electron donors and acceptors[29] (size proportional to the inferred availability). Hd, Hadean; Ph, Phanerozoic.

composition of organic carbon-rich shales from 2.66 to 2.08 Ga has been shown to reflect changes in weathering processes[62], indicating biological copper use before and after the GOE.

As global oxygenation expanded, copper became widely exploited in aerobic pathways, including the direct access of O_2 as an electron sink for respiration[2,6,63,64]. Indeed, the availability of copper allowed access to the highest-redox-potential half-cells used by life, thereby extracting more energy from carbon metabolites[7]. Copper protein functions include electron transfer, O_2 binding, O_2 activation and reduction to water, NO_2^- and N_2O reductions, and substrate activation such as hydrogen atom abstraction[65–67]. The active sites of copper proteins have been divided into three classes based on geometry: type 1 is blue copper, type 2 normal copper, and type 3 coupled binuclear copper centres[68–70]. Reduction potentials for type 1 copper protein can range from +183 to +800 mV (refs 71,72). This is not only a very large range but also a highly positive range which is difficult for iron redox chemistry to achieve, allowing for expanded substrate specificity with copper proteins[8]. The high reduction potentials of copper proteins are required to reduce strong oxidants such as O_2 and N_2O (refs 2,72,73). Copper protein reduction potentials are also highly tunable, depending on different axial ligands: glutamine, +190 to +320 mV; methionine, +183 to +670 mV; multi-copper non-coordinating phenylalanine/leucine/threonine, +354 to +800 mV (refs 74–83).

Copper chemistry in biology is largely constrained to Cu(I)/Cu(II) one-electron redox reactions. The electron configuration of copper is $[Ar]4s^13d^{10}$ (Supplementary Table 3), allowing *s*-orbital bonding at low oxidation states. Both Cu(I) and Cu(II) ions can bind tightly to organic ligands, such as amino acid side-chain N and S groups (that is, histidine, cysteine), leading to efficient trapping of copper compared with other metal cofactors[8]. For monovalent ions, the binding stability constant, *K*, of Cu^+ is much higher than for the biologically common ions Na^+ and K^+ (that is, $Cu^+ >> Na^+, K^+$). For divalent ions, Cu^{2+} is still at the top of the stability series: $Cu^{2+} > Zn^{2+}$, $Ni^{2+} > Co^{2+} > Fe^{2+} > Mn^{2+} > Ca^{2+}$, Mg^{2+} (ref. 8). Copper thus provides a source of one-electron couples of highly positive potentials (up to +800 mV) while maintaining higher-stability complexes with nitrogen or sulfur ligands in their low oxidation states than other transition metal centres[84]. The combination of access to high redox potentials, better substrate specificity, efficient trapping/stability and higher bioavailability made copper the ideal metal to be used in aerobic metabolisms as the planet became oxygenated.

The emergence of aerobic microbes that replaced anaerobic microbial communities can be observed in the relatively high (approximately 10‰) ^{13}C enrichment in organic carbon from shallow sediments relative to deep-water sediments at 2.72 to 2.45 Ga in the Hamersley Province, Western Australia[85]. The enrichment in ^{13}C probably represents a decreased influence of the assimilation of methane or other ^{13}C-depleted substrates. This is around the same time that geochemical evidence of other copper-utilizing metabolic pathways can be observed (Figs 2, 3). The evolution of nitrogen fixation (3.2 to 2.9 Ga), oxygenic photosynthesis (3.0 to 2.5 Ga), methane oxidation (2.9 to 2.7 Ga),

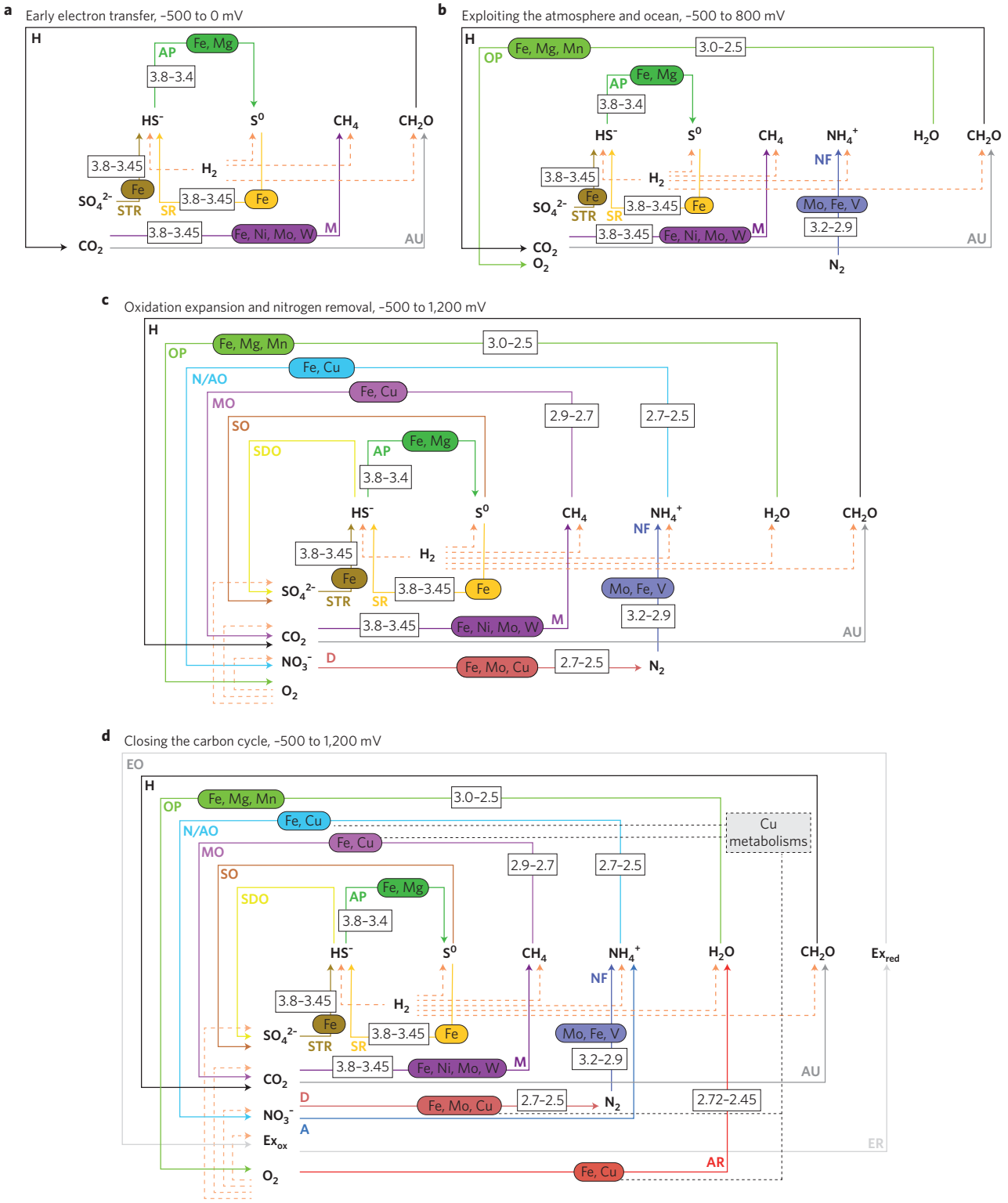

Figure 3 | Global electron-transfer network and redox potential expansion through time. The figure is based on preserved geochemical evidence ages of metabolisms from Fig. 2 and Supplementary Table 2. Figure redrawn and adapted, including redox potential ranges, from Falkowski *et al.*[1] and Jelen *et al.*[2]. Geochemical evidence ages in Ga are given in boxes. Molecules listed horizontally (HS^-, S^0, CH_4, NH_4^+, H_2O, CH_2O, Ex_{red}) are reduced substrates, molecules listed vertically (SO_4^{2-}, CO_2, NO_3^-, O_2, Ex_{ox}) are oxidized substrates, and N_2 is an intermediate and atmospheric reservoir of unreactive nitrogen. Solid lines connect redox couples in oxidation or reduction reactions resulting from each metabolism. Dashed lines represent physical participation of O_2 or H_2 (H^+) in the reaction rather than redox chemistry. Metals used in oxidoreductase proteins (EC1) associated with each metabolism are listed. A, ammonification; AP, anoxygenic photosynthesis; AR, aerobic respiration; AU, autotrophy; D, denitrification; EO, other elements oxidation; ER, other elements reduction (EO and ER include Fe and Mn oxidation and reduction); H, heterotrophy; M, methanogenesis; MO, methane oxidation/methanotrophy; N/AO, nitrification/ammonia oxidation; NF, nitrogen fixation; OP, oxygenic photosynthesis; SDO, sulfide oxidation; SO, sulfur oxidation; SR, sulfur reduction; STR, sulfate reduction. Figure reproduced from Ref. 2, Annual Reviews.

Figure 4 | Phylogenetic tree of the main lineages of Bacteria and Archaea and their putative divergence times. Divergence confidence intervals are drawn as scale bars around the respective nodes (represented by light-grey rectangles). The last common ancestor was arbitrarily placed at 4.25 Ga in the tree as previously described by Batistuzzi *et al.*[93]. Estimated divergence times were retrieved from Battistuzzi and Hedges[94] for Archaea, and Battistuzzi *et al.*[93] for Bacteria. The position of the first vertex of each polygon represents the phylogenetic distance of the first internal split within the taxa. The polygon area is proportional to the number of sequences present in the SILVA rRNA SSU database of type strains. Metabolism ages are given from Fig. 2 and Supplementary Table 2. Timescale: Hd, Hadean; Ph, Phanerozoic.

nitrification/denitrification (2.7 to 2.5 Ga) and aerobic respiration (2.72 to 2.45 Ga), which incorporated the available metallocofactors iron, magnesium, manganese, molybdenum and copper, represents the expansion of the global network of electron transfer and access to a larger range of positive redox potentials (−500 to +1,200 mV; Fig. 3). The presence of these metabolic pathways closed the nitrogen and carbon cycles before the oxygenation of the oceans and atmosphere, and evolution of diverse and specialized organisms. Chemotrophic Fe- and Mn-oxidation and reduction are represented among extra oxidation and reduction metabolic pathways (Ex_{ox} and Ex_{red}; Fig. 3; pathways are discussed in Supplementary Information).

Oxidoreductase metal usage and microbial evolution

As described above, the EC1 proteins of core microbial metabolisms incorporated particular metals at different time periods of the Archaean eon to catalyse electron transfer (Figs 2, 3; Supplementary Table 2). Owing to gene inheritance and HGT[27], metal usage in EC1 proteins also differs depending on microbial phyla. To analyse the relationships between oxidoreductase metallocofactors, metabolism and evolutionary history, we compiled metal cofactors of all EC1 proteins present in the Uniprot database for the main bacterial and archaeal lineages (Fig. 4). Protein cofactor annotations were incomplete in some cases, and we used manual correction from the literature to remedy inconsistencies (as described below for nitrogenase). We compared estimated divergence times from previous work[86,87] with geochemical evidence of metabolic pathways. We selected these works for the wide range of phylogenetic diversity included in the analyses. The limitations of molecular clock models (that is, changing generation times, species-specific differences, population size, change in protein function and changes in natural selection) should be considered when using these techniques[88,89].

Unsurprisingly, according to this analysis, iron is present in all representative taxa shown in Fig. 4. Across all taxa, EC1 metal utilization was found to be Fe > Mn, Mg > Ni > Mo > Cu > Co > W, V. Iron and

nickel are the most common EC1 metals in Euryarchaeota taxa, and are used in methanogenesis, sulfate reduction and sulfur reduction for these taxa (Fig. 4). Molybdenum is used in only two of these taxa, but nitrogen fixation is present in all representative Euryarchaeota taxa (Fig. 4), suggesting alternative forms of nitrogen fixation in Mo-free Euryarchaeota taxa. Crenarchaeota EC1 metals include molybdenum and copper which are used in nitrification, denitrification and aerobic respiration.

There is a clear distinction between bacterial taxa with low metal diversity and bacterial taxa with high metal diversity (Fig. 4). The number of EC1 metallocofactors in the 'low metal' bacteria (LMB) group ranges from 3 to 5 with an average of 3.75 per taxon. In contrast, the number of EC1 metallocofactors in the 'high metal' bacteria (HMB) group ranges from 4 to 10 with an average of 7 per taxon (Mg/Mn and Mn/Fe each count as one metallocofactor). Similarly, the number of core metabolisms among LMB ranges from 1 to 3 with an average of 1.6 per taxon, and the number of core metabolisms among HMB ranges from 2 to 7 with an average of 4.2 per taxon. In particular, molybdenum and copper are much more widespread in the EC1 proteins of the HMB group, resulting in the far greater presence of nitrogen fixation, methane oxidation, nitrification, denitrification and aerobic respiration in the HMB than in the LMB.

Generally, the diversity of EC1 metals increases in taxa with metabolic pathways that evolved more recently (Fig. 4). The diversity of EC1 metals and metabolisms increases from older to younger diverging taxa as well. This transition over time, including the evolution of HMB, increases the range of biologically accessible redox potentials for protein cofactors[2,9]. Protein-fold diversity also increases with metal diversity and specificity, as a protein-fold binding site that can accommodate magnesium or manganese (represented as 'Mg/Mn') is different from a fold that can use only one of these cofactors[9,90]. This is in agreement with previous work showing that protein architectures that can bind more than one metal evolved before metal-specific binding sites, and that the evolution of metal homeostasis protein structures coincided with the rise of metal-specific structures in the Archaean ocean[3].

The expanded EC1 use of molybdenum, copper and other metallocofactors in later branching HMB taxa reflects the emergence and dispersal of metabolisms including nitrogen fixation, methane oxidation, nitrification/denitrification and aerobic respiration from 3.2 to 2.45 Ga (Figs 3, 4). The Proteobacteria cluster and the cluster containing Actinobacteria, Deinococcus-Thermus, Cyanobacteria, Chloroflexi and Firmicutes exhibit this expanded use of molybdenum, copper and associated metabolisms. Use of molybdenum and copper in older branching taxa Aquificae and Crenarchaeota is potentially a consequence of HGT resulting in acquired nitrification and denitrification metabolisms and acquired systems for oxygen species detoxification. A recent analysis of the ancestral and acquired metabolic traits in the Aquificae has revealed that nitrate reduction, catalysed by the Mo-containing nitrate reductase, also seems to have been acquired by HGT in this group[91]. However, there is a limitation of database protein cofactor annotation that results in the absence of predicted Mo-containing EC1s in Chloroflexi and Chlorobi, which include species known to possess the *nifHDK* genes that code for Mo-containing nitrogenase[92]. With this molybdenum utilization added manually for Chloroflexi and Chlorobi, there is a stronger connection between molybdenum and nitrogen fixation among bacterial taxa (Fig. 4). Multiple Euryarchaeota taxa that are not shown to utilize molybdenum in the Uniprot database are able to fix nitrogen. This could support an origin for primitive, now extinct form(s) of Fe-nitrogenase-based nitrogen fixation before the observed geochemical evidence at 3.2 to 2.9 Ga.

An expanding network of geosphere–biosphere links

Previous bioinformatic studies have shown that metal availability and incorporation directly influenced biological innovation and evolution[3,17,23]. Specifically, the emergence of proteins that bind metal and facilitate electron transfer provides the foundation for an expansion of microbial metabolisms[1,3], resulting in the biogeochemical cycling of multiple nutrients and substrates. By synthesizing a wide range of evidence from the geochemical record with biological metal utilization and metabolism, we can reconstruct the early Earth's biogeochemical network and identify the links between global redox state, metal availability, metabolic pathways and microbial evolution (Figs 3, 4). Early organisms initially accessed negative redox potentials in the reducing Archaean ocean at 3.8 to 3.4 Ga using sulfur reduction, sulfate reduction, methanogenesis and anoxygenic photosynthesis. The evolution of nitrogen fixation between 3.2 and 2.9 Ga (or earlier) supported the expansion of microbial communities by providing a substantial source of fixed nitrogen. The network expanded to positive redox potentials as the ocean became oxidized, from 3.0 to 2.45 Ga, which allowed the exploitation of new metal cofactors and electron transfer reactions. This resulted in aerobic respiration of organic matter and the loss of fixed nitrogen through the coupling of nitrification to denitrification[49].

The use of solar energy by early microbes has historically provided the dominant source of energy for the biosphere[93]. The emergence of Cyanobacteria and oxygenic photoautotrophy between 3.0 and 2.7 Ga (refs 59,86,87,94,95) marked a turning point in the Earth's history: not only are Cyanobacteria predicted to use a much broader range of metals than the adjacent branching phylum, Chloroflexi, but the oxygen produced during these reactions altered the availability of other key metals. The subsequent evolution of metabolic pathways resulted in the closure of key biogeochemical cycles, and the formation of a complete circuit of biological electron transfer. This important change in biological activity and biogeochemical cycling highlights the importance of the co-evolution of the geosphere and biosphere in creating the electron flows that predominate today.

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Author contributions

E.K.M. (lead author) carried out data analysis; B.I.J., D.G. and H.R. contributed to data analysis and writing; P.G.F. (primary investigator) contributed to writing the paper.

Additional information

Supplementary information is available in the online version of the paper.

Competing financial interests

The authors declare no competing financial interests.