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Was nitric oxide the first deep electron sink?

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Evolutionary histories of enzymes involved in chemiosmotic energy conversion indicate that a strongly oxidizing substrate was available to the last universal common ancestor before the divergence of Bacteria and Archaea. According to palaeogeochemical evidence, O₂ was not present beyond trace amounts on the early Earth. Based on recent phylogenetic, enzymatic and geochemical results, we propose that, in the earliest Archaeal, nitric oxide (NO) and its derivatives nitrate and nitrite served as strongly oxidizing substrates driving the evolution of a bioenergetic pathway related to modern dissimilatory denitrification. Aerobic respiration emerged later from within this ancestral pathway via adaptation of the enzyme NO reductase to its new substrate, dioxygen.

Chemiosmosis in early life: the question of the terminal electron acceptor

Mitchellian chemiosmotic energy conversion [1], the process by which systems harvest the Gibbs free energy contained in the electrochemical disequilibrium between reducing and oxidizing substrates, is a common feature of Bacteria and Archaea. Therefore, it probably already operated in what is commonly referred to as the ‘last universal common ancestor’ (LUCA) of the two prokaryotic domains [2]. On modern Earth, strongly oxidizing substrates abound, whereas strongly reducing ones, such as molecular hydrogen (H₂) or reduced sulfur compounds, are confined to specific habitats. This predominant oxidizing environment results from oxygenic photosynthesis pumping O₂ into the biosphere for at least 2.5 billion years [3–7].

Before the onset of oxygenic photosynthesis the average redox state of the habitable environment must have been substantially lower with a likely predominance of H₂, reduced sulfur and ferrous iron [8,9], whereas the anthropocentrically ‘archetypal’ substrate to chemiosmosis (Figure 1), O₂, was almost certainly absent [10]. Recent hypotheses on the origin of chemiosmosis, therefore, invoke the relatively weak oxidant CO₂ as the most likely early electron acceptor from H₂, yielding acetate or methane as reaction products [2,11].

However, molecular phylogenies of several bioenergetic enzymes indicate the presence of bioenergetic electron

sinks that are substantially more oxidizing than CO₂ in the early Archaeal. Based on a re-evaluation of the phylogeny of the heme copper oxidase (HCO) superfamily and on recent insights in structure–function particularities of the major HCO subgroups, we argue that nitric oxide (NO) was the likely ancestral substrate to the HCO superfamily in the earliest Archaeal. Contrary to the case of oxygen, a source for mass production of NO and its derivatives (NO₃[−] and NO₂[−]) existed on the early Earth [12]. Our scenario suggests an important role for the dissimilatory denitrification pathway or of its constituent segments in primordial energy conversion. Aerobic respiration would have originated from within the denitrification pathway through adaptation of NO reductase to its new substrate, O₂.

Evolutionary histories of bioenergetic enzymes and the increasing environmental oxidation state

Enzyme phylogenies encompassing representatives from both prokaryotic domains, Bacteria and Archaea, serve to deduce whether a given enzyme existed before the Archaea–Bacteria split or appeared more recently. So far, only a few bioenergetic enzymes have been studied to this end. Phylogenies of Ni-Fe hydrogenase (i.e. the enzyme serving as the entry point for electrons from H₂ into energy-conserving chains) indicate that this redox complex evolved before the Archaea–Bacteria split [13,14], in agreement with the abundance of H₂ in specific habitats on the early Earth and with the resulting likely crucial role of hydrogenase in primordial bioenergetics [11]. An unexpected important role of arsenics as redox substrates in primordial habitats is becoming increasingly evident [15]. In the early Archaeal, the reduced form, arsenite, was most likely to be predominant and, correspondingly, the bioenergetic enzyme arsenite oxidase [Figure 2a(i)] seems to be pre-LUCA [16].

In turn, phylogenies of several enzymes that reduce high redox-potential substrates bear distinctive signs of late, that is, post-LUCA origins followed by dissemination via horizontal gene transfer. Prominent examples are provided by the complexes involved in electron transfer towards oxidized sulfur compounds [17] or in arsenate respiration [18] [Figure 2a(i)], which uses the oxidized form of arsenics as the terminal electron acceptor. Indeed, arsenate reductase seems to have appeared long after the Archaea–Bacteria divergence, probably driven by rising

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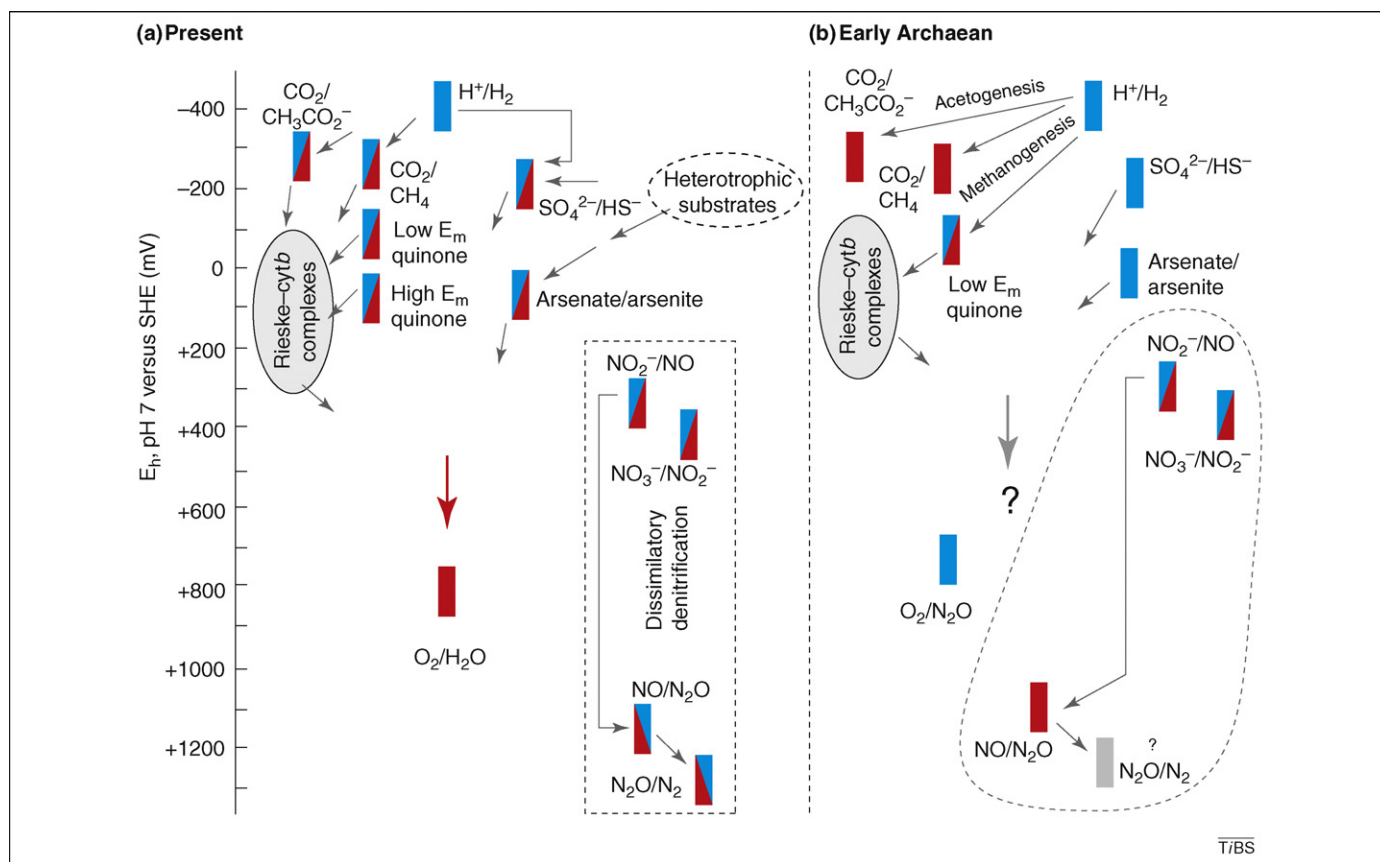


Figure 1. Energy-conserving redox reactions on the modern and the primordial Earth. Today, owing to the almost ubiquitous O_2 , the ambient oxidation state of the environment is high and a plethora of different bioenergetic electron-transfer chains operate in living organisms. Only a few examples pertinent in the context of our scenario are depicted in part (a). The vertical bars are centered at the standard midpoint potentials (E_m) of the bioenergetic substrates (versus the standard hydrogen electrode [SHE]) and extend to 60 mV on both sides of the E_m , to indicate the range of ambient potentials in which these chemicals change their redox states (from ~10% to 90% reduction; E_h , pH 7 stands for the ambient redox potential at pH 7). For simplicity, the differing ‘steepness’ of redox reactions of one-, two- or more-electron carriers was neglected. Blue or red boxes indicate that the corresponding redox couple is present predominantly in the reduced or oxidized state, respectively, whereas mixed colored boxes mean that the redox compound is oxidized or reduced according to the specific ambient conditions. The shaded ellipsoids mark the range of E_m values of redox cofactors harbored by the Rieske-cytb complexes. In the earliest Archaean environment, most modern electron acceptors are widely considered to have been fully reduced and, hence, are unavailable as electron acceptors (b). This would leave Archaean bioenergetics confined to electron transfer to CO_2 (i.e. acetogenesis and methanogenesis). However, as we argue here, several phylogenetic arguments point towards the presence of electron acceptors with E_m values >100 mV, raising the question of the chemical nature of such high-potential acceptors (indicated by ‘?’). NO and its derivatives, nitrate and nitrite, produced by electrical discharge and follow-up reactions from CO_2 and N_2 (Box 1), are likely to have played the part of strong oxidants on the early Earth. Whether an enzyme related to nitrous oxide reductase might have closed the cycle towards atmospheric N_2 remains an open question.

arsenate levels induced by the increasing environmental oxidation state.

As logical as the dissimilar evolutionary histories of arsenite oxidases and arsenate reductases might seem, considering the gradual oxidation of substrates by photosynthesis, a nagging problem persists. Arsenite oxidase oxidizes a substrate with an electrochemical potential of approximately +60 mV at pH 7 (Figure 1). For this reaction to be productive in a bioenergetic chain there must be a terminal oxidant. This substance should be substantially more oxidizing than arsenate. According to its phylogeny, arsenite oxidase seems to have functioned before the Archaea-Bacteria split, that is, long before photosynthetic oxygen production [3,5] set in; therefore, the nature of such a terminal oxidant remains enigmatic (Figure 1).

The substrate conundrum of the Rieske-cytb complexes

Another enzyme that strikingly violates the rule that high redox-potential substrates correlate with post-LUCA emergence is the Rieske-cytochrome *b* (*cytb*) complex. This enzyme is almost ubiquitous among prokaryotes and plays a pivotal part in energy conversion [19]. The family is widely

known through two specific representatives, the *cyt bc₁* complex (corresponding to mitochondrial complex III) and the *cyt b_{6f}* complex operating in cyanobacteria and chloroplast oxygenic photosynthesis. The phylogenetic trees of both cytochrome *b* and the Rieske protein strongly resemble that of common 16S ribosomal (r)RNA trees and, in particular, show a pronounced cleavage into archaeal and bacterial subtrees [20]. It is possible to root the tree because the Rieske protein is also an arsenite oxidase subunit. The composite phylogeny of Rieske-cytb complexes and arsenite oxidases [16,20] strongly indicates a pre-LUCA origin for both enzymes and, thus, corroborates the corresponding conclusion drawn for the catalytic molybdenum subunit of arsenite oxidases [Figure 2a(ii)].

The Rieske-cytb complexes transfer reducing equivalents from quinols to soluble electron-shuttle proteins such as cytochromes, copper proteins or high-potential iron sulfur proteins, which, in turn, reduce terminal oxidases. The midpoint potentials of these electron shuttles admittedly vary as a function of the chemical nature of the quinone molecule [21] but always are in the positive range of potentials (Figure 1). To efficiently pull electrons

through the chain, the terminal electron-accepting substrates are expected to be substantially more oxidizing with redox potentials no lower than +100 mV.

The apparent pre-divergence origin of the Rieske–cyt b complexes, as for arsenite oxidase, implies the availability of a strong oxidant in the early Archaeon. Guided by the layout of extant bioenergetic mechanisms [Figure 1(a)], molecular oxygen would be the straightforward candidate for such an oxidizing molecule. Although most paleochemical proxies indicate extremely little or no O $_2$ in the early Archaeon [10], there are counter suggestions in favor of oxic intervals in this era [22].

From the point of view of molecular evolution, it is tempting to tackle the question of molecular oxygen ancestry by analyzing the phylogeny of the enzymes that use O $_2$ as their (bioenergetic) substrate, that is, the respiratory O $_2$ reductases, historically called cytochrome (and quinol) oxidases.

HCO superfamily phylogeny: the controversy

Three major groups of respiratory O $_2$ reductases have been identified [23] and are referred to as HCOs based on their cofactor composition comprising heme groups and copper atoms. A fourth group belonging to this enzyme superfamily was recognized in 1994 based on substantial similarities in sequence and cofactor composition [24]. The members of this additional group reduce NO rather than oxygen and they deviate from the O $_2$ reductases owing to the presence of an iron atom, rather than a copper ion, in the catalytic site. In a series of articles some 15 years ago, Matti Saraste and colleagues concluded, from the prominent Archaea–Bacteria cleavage in phylogenetic trees of HCOs, that HCOs had their origin in the common ancestor of Bacteria and Archaea [25–27]. A possible descent of O $_2$ reductases from NO reductases was considered based on the evident affiliation of the latter enzymes to the HCO superfamily [28]. These results thus favored the scenario of O $_2$ being present before the divergence of domains.

Whereas the early phylogenetic trees were reconstructed from only a handful of sequences, a more recent study analyzing a larger sample of dioxygen reductase sequences arrived at the opposite conclusion [29]. Pereira *et al.* [29] suggest that lateral gene transfer occurred among many early-diverging branches of the tree. One of the three groups, the cyt cbb_3 oxidases, were found to be devoid of archaeal representatives and seemed to contain only proteobacterial species leading to the proposal that this group diverged more recently from one of the other two O $_2$ reductase groups [29,30]. The ensemble of these observations was taken to indicate a post-divergence origin of dioxygen reductases followed by distribution over the entire prokaryotic world via lateral gene transfer.

HCO superfamily phylogeny: pre- and post-dualism

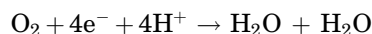
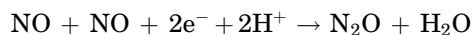
We have reanalyzed the phylogeny of the HCO superfamily (including the related heme-iron NO reductases) taking advantage of the recent explosion in the number of sequenced prokaryotic genomes. This tree [Figure 2a(iii)] features several well-defined clades. Two of these clades arise from quinol- and cytochrome-oxidizing NO reductases (qNOR and cNOR), respectively. The qNOR

clade divides almost perfectly into archaeal and bacterial subtrees. The clade representing cbb_3 oxidases diverges close to NORs, as already noticed by Castresana and Saraste [26]. In our analysis, the cbb_3 clade was well separated from all other clades and encompassed nearly the full tree of the domain Bacteria, refuting the notion of a recent divergence of this enzyme from other types of HCO [31]. However, no archaeal cbb_3 family members were detected. The remaining clades of dioxygen reductases contain archaeal and bacterial representatives but, although well-separated archaeal and bacterial clusters are present, the roots do not fall in between Archaea and Bacteria [Figure 2a(iii)], in line with the results of Pereira *et al.* [29]. These phylogenies, thus, indicate early inter-domain gene transfer (except for the cbb_3 oxidases) and subsequent vertical inheritance for the different groups of O $_2$ reductases rather than pre-divergence origins.

As much as this conclusion seems to be straightforward for each individual O $_2$ reductase clade, the structure of the whole tree leads to an entirely different conclusion. All HCO groups (including NOR) form well-defined clades separated by long rooting branches. This topology is difficult to rationalize assuming a single origin of a dioxygen reductase in either Archaea or Bacteria. Moreover, the assumption of a pre-divergence origin for qNOR, as indicated by the phylogenetic tree, implies that the common ancestors of the O $_2$ reductase clades, all being outgroups to NOR, already were present in the common ancestor. The structure we obtain for the phylogenetic tree of the HCO superfamily, therefore, gives contradictory messages, supporting at the same time a pre- and a post-divergence origin of HCOs. This tree, thus, remains incomprehensible in the absence of a radically new evolutionary paradigm.

From NO to O $_2$ reductases: the problem of the missing electron

Despite the close phylogenetic relationship and the resulting structural similarities of the enzymes involved, the biochemical reactions of NO and O $_2$ reduction differ substantially [32,33]. Reduction of NO to nitrous oxide is a bimolecular reaction combining two NO molecules to N $_2$ O and water, whereas in dioxygen reduction a single O $_2$ molecule yields two water molecules:



Furthermore, twice the number of reducing equivalents is required for O $_2$ compared with NO reduction.

In HCO-type O $_2$ and NO reductases, the catalytic step occurs in the binuclear center, which comprises a 5-coordinated heme moiety and a copper or an iron atom (Figure 2b). O $_2$ and the two NO molecules are assumed to bind between the two metal centers. The two reducing equivalents necessary for the conversion of two NO molecules into N $_2$ O are individually delivered by ferrous (Fe $^{2+}$) to ferric (Fe $^{3+}$) transitions of the heme and the non-heme irons in the binuclear center of NO reductases. In O $_2$ reductases, the heme shuttles between the ferrous (Fe $^{2+}$) and the ferryl (Fe $^{4+}$) states, thereby contributing two electrons to the catalytic step. The biologically accessible redox

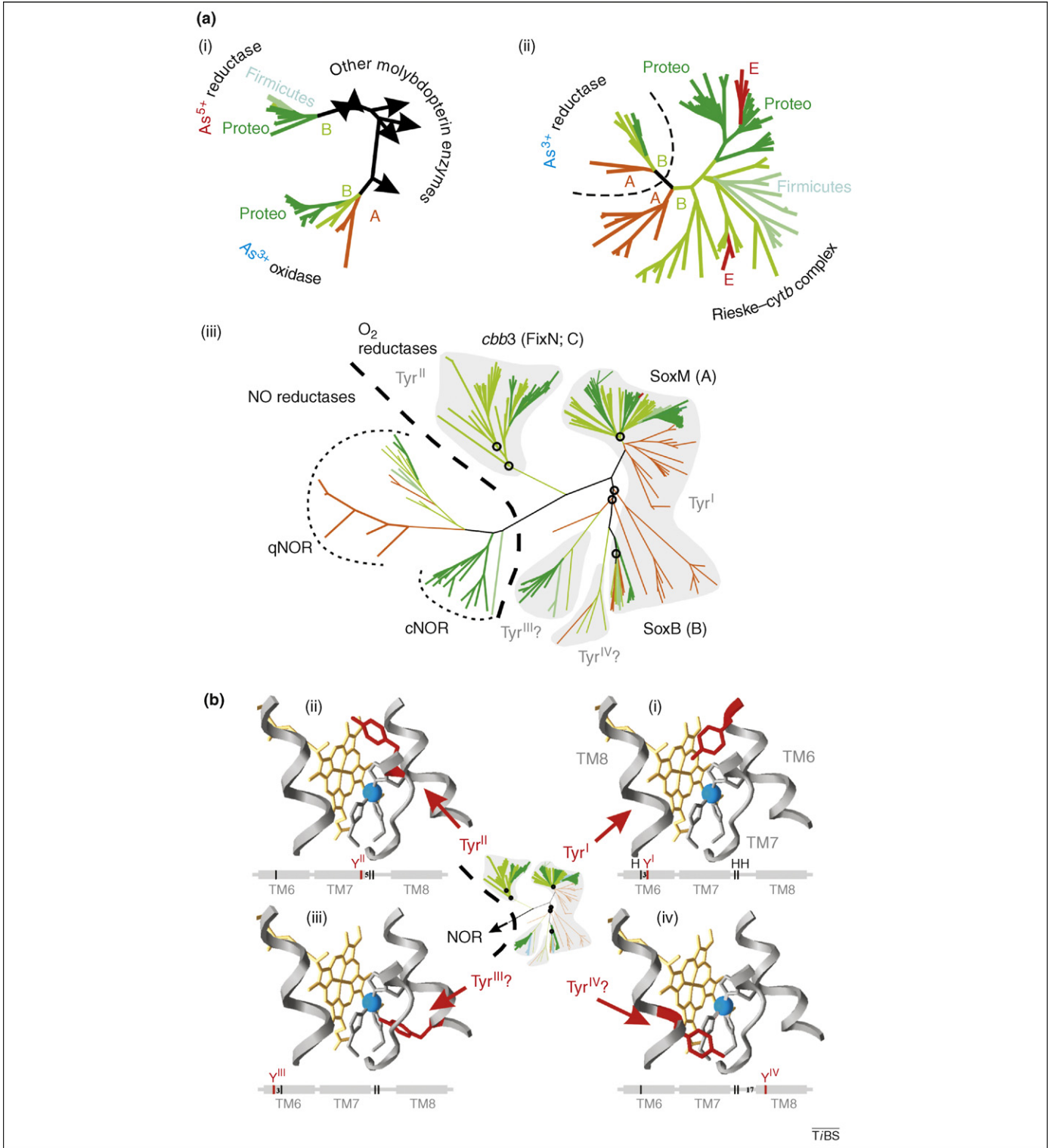


Figure 2. Molecular phylogenies of selected bioenergetic enzymes together with structural idiosyncrasies of different HCO clades indicate independent origins of O_2 reductases from NOR. **(a)** Shown are molecular phylogenies reconstructed on (i) the molybdopterins catalytic subunit of arsenate (As^{5+}) reductase, arsenite (As^{3+}) oxidase and other enzymes from this superfamily [18], (ii) the Rieske subunit of arsenite oxidase and the Rieske-cytb complex [20] and (iii) the catalytic subunit of representative members of the HCO superfamily. The topologies of the individual trees provide information on the evolutionary history of the respective families. Enzymes occurring in Archaea (A) are coded by orange branches, whereas those harbored by Bacteria (B) are marked in cyan (Firmicutes), dark green (Proteobacteria [Proteo]) and light green (all others). Eukaryotic (E) representatives are indicated in red. Trees featuring distinct Archaea-Bacteria cleavages at their roots indicate pre-divergence origins of the corresponding enzyme. This is, for example, the case for the Rieske-cytb complexes (ii) and arsenite (As^{3+}) oxidase (i and ii). By contrast, trees characterized by the absence of this cleavage and/or strong incongruence with 16S rRNA-based species trees (such as observed for the As^{5+} reductase subtree) indicate late origins and dissemination via lateral gene transfer. The tree reconstructed on the HCOs (iii) is ambiguous with respect to these criteria and can, therefore, not be interpreted conventionally. In the HCO tree, qNOR and cNOR stand for quinol- and cytochrome-oxidizing NO reductases, respectively. The denomination of O_2 reductase clades (shaded) features both the nomenclature used by Saraste and colleagues [25] (i.e. SoxM, SoxB and FixN) and the more recent one introduced by Pereira *et al.* [29] (A-, B- and C-type). The phylogenetic tree has been reconstructed using the neighbor-joining method. Nodes supported by bootstrap values <50% are marked by circles. The topology of the tree at these nodes, therefore, is less reliable than in the rest of the tree. A detailed version of the tree featuring species names and its underlying alignment are available on request. **(b)** O_2 reductases distinguish themselves from NO reductases by the presence of a catalytically important tyrosine residue close to the active center. The catalytic binuclear center

couple of the copper ion only enables the Cu^+ to Cu^{2+} transition, adding a third electron to the reaction. This leaves the binuclear center short of one electron to proceed towards full reduction of O_2 . A multiple-step reduction of dioxygen (i.e. involving re-reduction of the heme in the binuclear center by secondary electron donors), however, presumably is evolutionarily strongly counter-selected against. Such a reaction scheme would produce reactive oxygen species (ROS) capable of diffusing out of the catalytic center and wreaking havoc in the parent cells. Indeed, in the well-characterized A (or SoxM)-type O_2 reductase (the enzyme functioning in our mitochondria) [Figure 2b(i)], the fourth electron is delivered (together with a proton in the form of a hydrogen atom) by the oxidation of a tyrosine residue [Figure 2b(i), labeled Tyr^I] present in the catalytic site [34–36].

Has the ‘tyrosine solution’ to the missing-electron problem been found independently several times?

In the past, the case of the *cbb*₃ oxidases frequently was used to argue against an indispensable role of the tyrosine in catalyzing HCO-mediated O_2 reduction. The tyrosine residue, which is fully conserved in SoxM-type oxidases, is indeed absent in *cbb*₃ oxidases. In 2005, however, a milestone article from Bob Gennis’ group [37] demonstrated that the tyrosine residue (Tyr^{II}) involved in *cbb*₃ oxidase catalysis is located in a different sequence position and even on a different helix [Figure 2b(ii)]. Despite it being distant on the primary sequence of the protein, molecular modeling indicated that Tyr^{II} is located in close vicinity to the binuclear center [37], occupying a position almost identical to that of Tyr^I [Figure 2b(i)]. The functional equivalence of this residue to that of the previously studied classes of O_2 reductases was subsequently shown by several laboratories [38,39]. These publications referred to the differing position of the tyrosine residue as ‘migration of a functionally important residue’. Mechanistically, a mutant strain having lost the tyrosine in one place would have inserted this residue in another sequence position to suppress the deleterious ROS production. Such double-site suppressor mutations have been demonstrated for many other enzyme systems [40].

However, in the light of the ensemble of observations and conundrums outlined earlier, another scenario seems much more likely to us because it solves in one sweep most of the riddles of the evolutionary history of HCO. We propose that, rather than resulting from migration, the differing positions of the tyrosine in the HCO oxidases, in fact, betray the independent origins of these O_2 reductases from separate, now extinct, NO reductase subgroups. In

this scenario, NO reductases would have diversified into several distinct groups before the Archaea–Bacteria divergence, much as it seems to be the case for the Ni-Fe hydrogenases [13,14]. With increasing O_2 concentrations in the environment, dioxygen became a potent terminal acceptor for bioenergetic chains and some of these NO reductases evolved into dioxygen reducing enzymes ‘simply’ by inserting an electron-donating amino acid residue in close vicinity of the catalytic site.

One specific circumstantial observation resulting from our study of the HCO phylogeny lends support to this evolutionary scenario. In these phylogenies [Figure 2a(iii)], two clades lack a tyrosine residue in both the Tyr^I and the Tyr^{II} sequence positions. Screening the sequence stretches that structurally come close to the catalytic center, we found tyrosines, conserved within each clade, denoted Tyr^{III} and Tyr^{IV}, respectively, at two further sequence positions. Molecular modeling of these enzymes yields three-dimensional (3D) positions for Tyr^{III} and Tyr^{IV} with distances to the binuclear center that are perfectly comparable with those of the two characterized cases (Figure 2b). None of these enzymes, lacking both of the ‘traditional’ tyrosines, has been studied and we, therefore, cannot be sure whether they correspond to O_2 reductases rather than to ‘reverted’ NO reductases such as the enzyme in *Bacillus azotoformans* [41]. A thorough analysis of selected members of these groups will help to elaborate our scenario in more detail – or possibly weaken its likelihood.

Accepting the idea of several independent origins for the ability to reduce dioxygen rationalizes the topology of the HCO tree in an intriguingly simple manner. This evolutionary scenario, based on phylogenetic and biochemical evidence, predicts that NO was the primordial substrate of HCOs. It is noteworthy that the NO– N_2O couple is even more oxidizing than is the O_2 – H_2O couple (Figure 1). However, we then must ask where the NO might have originated on the early Earth.

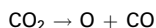
Large-scale production of NO and its derivatives in the primordial atmosphere

Several processes as diverse as lightning, volcanism and meteorite impacts would have produced up to 3×10^{12} g/yr of NO in the Archaean (Box 1). Part of this NO would subsequently have been photochemically converted (via HNO) to nitrate and nitrite and rained out into the primordial ocean (Box 1). The main substrates of the ‘modern’ dissimilatory denitrification pathway were, thus, present in the Archaean biosphere and probably provided the evolutionary driving force for the appearance of the denitrifying chain of enzymes or segments thereof. The NOR

of O_2 reductases is made up by a heme group (yellow), a copper ion Cu_B (blue) with its three histidine ligands and the redox-active tyrosine (red). Stretches of three nearby transmembrane (TM) helices (TM6 to TM8) are also shown in this figure. Four types of catalytic sites are grouped around the phylogenetic tree of O_2 reductases [reproduced in a thumbnail version from panel (a)]. (i) This structure is based on the crystallographic 3D structure of a SoxM-type O_2 reductase (PDB ID code 1V54; www.rcsb.org/pdb) and served as a template for the three remaining structures for which no 3D information is yet available. Although positioned on TM7 rather than TM6 (i.e. the canonical position in all SoxM-type O_2 reductases), the catalytic tyrosine of *cbb*₃-type enzymes (ii) comes structurally as close to the heme-copper diad as does the tyrosine residue in (i) [37]. The two different sequence positions for this tyrosine residue observed in SoxM- and *cbb*₃-type O_2 reductases [37] are denoted Tyr^I and Tyr^{II}, respectively. In the HCO tree [see part (a) of this figure], we observe two clades that contain neither Tyr^I nor Tyr^{II} but instead feature tyrosines in two new sequence locations (Tyr^{III} and Tyr^{IV}). The structural models (iii and iv) of the catalytic site of enzymes possessing Tyr^{III} and Tyr^{IV} indicate that these tyrosines are again spatially close to the catalytic center and might, therefore, have a functional role similar to that of Tyr^I and Tyr^{II}. Because no representatives of the Tyr^{III} and Tyr^{IV} groups have been studied so far, these putatively important tyrosines are labeled with a ‘?’. The differing positions of the catalytic tyrosines can be rationalized assuming independent origins of the various O_2 reductase clades from ancestral NO reductases, which in turn abolishes the phylogenetic ambiguity of the HCO tree. The schematic representation of the transmembrane helices TM6 to TM8 beneath each structure indicates the corresponding positions of the respective tyrosine residues (red bar) and the three conserved histidine ligands to Cu_B (black bars). The numbers of residues between each of these tyrosines and the closest histidine residue are shown.

Box 1. Nitrogen oxides from CO₂, N₂ and energy

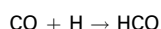
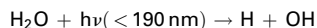
As shown experimentally [12], electrical-discharge events in an atmosphere containing CO₂ and N₂ produce NO via the following chain of reactions:



Several sources for high-voltage electrical discharge existed in the Archaean and would have added up to create NO in the atmosphere.

- Volcanically induced lightning would have produced $\sim 6 \times 10^{11}$ g/yr [44].
 - Meteorite impact plumes are estimated to have generated $\sim 10^{12}$ g/yr [45].
 - Thunderstorm lightning would have yielded another $\sim 3 \times 10^{11}$ g/yr [12].
 - Martin *et al.* [46] have, furthermore, argued that high temperature alone in volcanic gasses would have created $\sim 1.5 \times 10^{12}$ g/yr.
- (a) to (d) add up to a total of $\sim 3 \times 10^{12}$ g/yr.

A small part of this atmospheric NO partitions directly into the ocean, whereas the bulk of this NO reacts, in the presence of water vapor, UV-photochemically to HNO according to the following reactions [43,47].

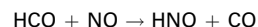


substrate would, thus, have been provided by the earlier steps of the denitrification cascade and possibly also by locally high NO concentrations directly produced in the electrical-discharge events.

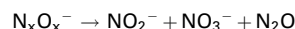
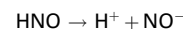
Conclusion

Our hypothesis stipulates that, contrary to the widely accepted dogma, a strong oxidant was present during the earliest Archaean. This oxidant was not O₂ but NO and its derivatives nitrate and nitrite, produced geochemically in substantial amounts. Navarro-González *et al.* [12] estimated that decreasing CO₂ concentrations in the late Archaean prompted dwindling NO production. This might have induced a 'nitrogen crisis for Archaean life' possibly triggering the appearance of biological nitrogen fixation mechanisms [12]. In the framework of our hypothesis, a reduced availability of nitrate, nitrite and NO would, in addition, have entailed an energy crisis, providing a further evolutionary driving force for the NO reductases of the denitrifying pathway to use the emerging O₂ as a new substrate.

Aerobic respiration, the quintessential bioenergetic mechanism of all (non-photosynthetic) complex life on this planet would, therefore, have its roots in an ancient energy-conserving mechanism closely related to modern dissimilatory denitrification. Using the geochemically produced, strongly oxidizing nitrogen oxides as electron acceptors enabled this mechanism to operate on weak reductants (e.g. the heterotrophic substrates [Figure 1] pyruvate or lactate) as electron donors, an option previously considered inaccessible to life before the advent of O₂. As a corollary to this fact, heterotrophic metabolism might also be much older than anticipated. Phylogenetic analyses of additional enzymes involved in these types of metabolism (see, for example, Ref. [42]) will provide insights into the details of Archaean bioenergetic mechanisms.



HNO rains out into the Hadean Ocean where it yields nitrate, nitrite and nitrous oxide [47] and where NO₃⁻ and NO₂⁻ (produced in a 4:1 ratio) [47] accumulate in the ocean and N₂O escapes back to the atmosphere.



The mass of the modern ocean amounts to 1.410²⁴ g [48]. The Hadean Ocean might have had up to twice that mass [48] and for the following we, therefore, assume a total of 3 × 10²⁴ g for the surface water on the early Earth. Because hydrological cycling between atmosphere and ocean is, and was, at least an order of magnitude more rapid than hydrothermal cycling between crust and ocean (which results in loss via reduction of the nitrogen oxides) [49] (<http://oceanworld.tamu.edu/resources/oceanography-book/hydrocycle.html>), accumulation of these nitrogen oxides to micromolar concentrations on average (and probably locally to even higher concentrations) would have taken approximately 1 million years, a mere blink of the eye on a geological timescale.

We would like to emphasize that the conceptual framework of the 'enzymatic' portion of our hypothesis was provided initially in 1998 by Matti Saraste and colleagues [28] who recognized the likely evolutionary ancestry of NO reductase over O₂ reductases. However, they proposed that both NO and O₂ reductases were pre-divergence enzymes. Ten years earlier, Mancinelli and McKay had, based on palaeo-geochemical arguments, arrived at the conclusion that dissimilatory denitrification probably operated in the Archaean, whereas aerobic respiration must have appeared only much later [43].

The scenario that we present here and that is based on the recent advances in the understanding of O₂ reductases merges these two visionary proposals while correcting a few details. Pre-divergence denitrification, indeed, seems to pre-date O₂-dependent aerobic respiration. This latter mechanism, however, directly arose from the evolutionary switch of the substrate for NO reductases: from NO to OO.

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