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The evolution of respiratory O_2/NO reductases: an out-of-the-phylogenetic-box perspective

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Complex life on our planet crucially depends on strong redox disequilibria afforded by the almost ubiquitous presence of highly oxidizing molecular oxygen. However, the history of O_2-levels in the atmosphere is complex and prior to the Great Oxidation Event some 2.3 billion years ago, the amount of O_2 in the biosphere is considered to have been extremely low as compared with present-day values. Therefore the evolutionary histories of life and of O_2-levels are likely intricately intertwined. The obvious biological proxy for inferring the impact of changing O_2-levels on life is the evolutionary history of the enzyme allowing organisms to tap into the redox power of molecular oxygen, i.e. the bioenergetic O_2 reductases, *alias* the cytochrome and quinol oxidases. Consequently, molecular phylogenies reconstructed for this enzyme superfamily have been exploited over the last two decades in attempts to elucidate the interlocking between O_2 levels in the environment and the evolution of respiratory bioenergetic processes. Although based on strictly identical datasets, these phylogenetic approaches have led to diametrically opposite scenarios with respect to the history of both the enzyme superfamily and molecular oxygen on the Earth. In an effort to overcome the deadlock of molecular phylogeny, we here review presently available structural, functional, palaeogeochemical and thermodynamic information pertinent to the evolution of the superfamily (which notably also encompasses the subfamily of nitric oxide reductases). The scenario which, in our eyes, most closely fits the ensemble of these non-phylogenetic data, sees the low O_2-affinity SoxM- (or A-) type enzymes as the most recent evolutionary innovation and the high-affinity O_2 reductases (SoxB or B and cbb_3 or C) as arising independently from NO-reducing precursor enzymes.

1. Introduction

It is undeniably the abundant and almost ubiquitous presence of the very strong oxidant O_2 that made complex life on the Earth thermodynamically possible. Hooking up the reducing end of biological (chemiosmotic) electron transfer chains to this powerful positive electrode of the planetary redox battery is achieved through the action of the so-called respiratory O_2 reductases (*alias* cytochrome/quinol oxidases or Complex IV). Respiratory O_2 reductases are membrane-integral redox enzymes coupling the $4e^-$ reduction of O_2 yielding two molecules of water to the translocation of up to eight [1,2] protons across chemiosmotic membranes. Its role as coupling agent between the environmental redox substrate O_2 and bioenergetic electron transfer chains makes respiratory O_2 reductases the ideal bio-proxy for shining light on the history of molecular oxygen on the planet Earth. As a result, respiratory O_2 reductases became targets for evolutionary studies over the last two decades [3–7], and they were among the very first bioenergetic enzymes to be analysed by

phylogenetic methods. However, in these early days, prior to the advent of large-scale genome sequencing, only a handful of amino acid sequences were available. Nevertheless, it had already been recognized that the respiratory O_2 reductases belonged to a larger superfamily of structurally strongly related enzymes which also included the nitric oxide (NO) reductases (NORs). NORs were first characterized from denitrifying bacteria, i.e. organisms using the oxidizing power of environmental nitrate and/or nitrite as the positive electrode for their bioenergetic electron flow. NORs, just as their sister enzymes the O_2 reductases, come in two distinct functional flavours, that is, those that oxidize soluble electron carriers such as cytochromes, copper proteins or high potential iron sulfur proteins (HiPIPs) on the electropositive (in prokaryotes the outside-) face of the membrane and a second group that receives the electrons required for NO (or O_2) reduction from membrane-diffusing quinol molecules.

From the very beginning, evolutionary approaches therefore attempted to produce phylogenetic trees integrating the complete superfamily of O_2 and NO reductases. As a result of the explosion of genomic data on organisms increasingly covering the (so far known) full diversity of life, these trees have grown new branches at breathtaking speed over the last decade [8–15]. Reassuringly, the general topology of these trees was only marginally influenced by the ever-increasing number of considered sequences, apart from the very early pioneering studies where the small number of available sequences precluded seeing the full extent of the superfamily's diversity. Even more comforting from the methodological point of view is the fact that the general topology of the tree appears insensitive to both the choice of tree-building algorithm and the geolocalization coordinates of the groups engaged in this research field [8,9,11,15].

One might therefore assume that the elucidation of the superfamily's evolutionary history is a done deal by now. Not at all! A number of evolutionary scenarios have been deduced from this tree that yield mutually exclusive predictions for the history of O_2 on our planet and of its role in biological energy conversion. This diversity of scenarios clearly cannot be blamed on the scientific quality of the individual research groups but is the fault of the tree itself. As has been pointed out in the past [11,12,15], this tree cannot be interpreted straightforwardly and it actually sends contradictory messages (see §4). Different groups have taken different routes to overcome the inherent bizarreness of the O_2/NO reductases' phylogeny resulting in contradictory and mutually exclusive scenarios. Aerobic respiration is thus proposed to have been operating in the last universal common ancestor (LUCA) of cellular life in one scenario [1,12,13], whereas others favour the later emergence of O_2 respiration from within an anaerobic type of respiration such as the denitrifying pathway [7,11]. Having provided opposite retrodictions for the evolutionary history of respiration from the same basic dataset, molecular phylogeny as the unique tool for solving this question has obviously failed.

A few years ago, Pereira & Teixeira [16] initiated the very promising integration of structural, functional and phylogenetic approaches to this problem. In this review article, we try to push this approach further by putting together an inventory of currently available information on the O_2 and NO reductases which might be pertinent to evolutionary questions and can complement the hard-core molecular phylogeny. This inventory includes three-dimensional structural information, recent data on functional idiosyncrasies, palaeogeochemical results as well as thermodynamic considerations. Even if all these non-phylogenetic perspectives do not yet allow us to arrive at an unambiguous solution, they might help to clear the logjam of molecular phylogeny.

2. No enzyme is an island

The vast majority of bioenergetic enzymes, just as is the case for enzymes in general, catalyse single steps in a larger chain or network of metabolically relevant reactions. The occurrence of properties in a given enzyme therefore crucially depends on the details of its integration into the system it is operating in. If this is true for any enzyme, it holds especially true for a bioenergetic complex for which not only physical reaction partners but also thermodynamic boundary conditions (which depend on the reaction partners) may vary wildly if integrated in different chemiosmotic chains (which might look deceptively similar at first glance). For the case of the O_2/NO reductases, the redox driving forces substantially differ between those that oxidize soluble carriers on the positive (P-) side of the membrane and those that oxidize liposoluble quinones. Within this latter group, there again are substantial differences if the pool quinones are high potential (such as ubi-, plasto- or caldariella-quinone, UQ, PQ or CQ) or low potential menaquinones (MK) or possibly quinones of intermediate potential (such as demethylmenaquinone, DMK). Some of the authors of this article [17,18] as well as others [19] have in the past argued that the low potential menaquinones likely evolutionarily preceded the higher potential quinone types. On their reducing side, members of the superfamily again deal with substrates of potentially greatly varying electrochemical potentials, that is, the NO/N_2O couple with a standard redox potential at pH7 ($E^{o\prime}$) of +1200 mV and the O_2/H_2O couple featuring $E^{o\prime}$ of +820 mV. Still, many (if not most) bioenergetic systems operate at conditions far from the standard ones entailing effective redox potentials (E_h) deviating by several hundreds of millivolts from $E^{o\prime}$ as will be detailed in §10.1.

Before investigating the details of structure and thermodynamics, we therefore first summarize (so far recognized) ways biology has found to make use of the structural/functional unit of the enzyme superfamily.

In the field of NORs, the enzyme drawing the two electrons required for reduction of two NO molecules to N_2O and water from soluble electron carrier molecules (mostly cytochrome *c* but likely also cupredoxins or HiPIPs) are denoted as cNORs as opposed to qNORs which oxidize membrane-soluble quinols. For simplicity and for the sake of emphasizing analogies, we will employ the same labelling scheme also for O_2 reductases (O_2Rs) throughout this article. What is generally referred to as 'cytochrome oxidase' in the dedicated literature will therefore here appear as cO_2R, whereas qO_2Rs correspond to the 'quinol oxidases' referred to by the 'haem–copper oxidase' community. Needless to say, we will try to avoid the term 'haem–copper oxidase' altogether for obvious reasons: NORs appear to contain iron rather than copper in their catalytic binuclear centre (BNC in Marten Wikström's terminology) and, as pointed out before [8], enzymes are fittingly named by their substrate conversion reaction, which is O_2 and NO reduction for the case of the two parts of the superfamily.

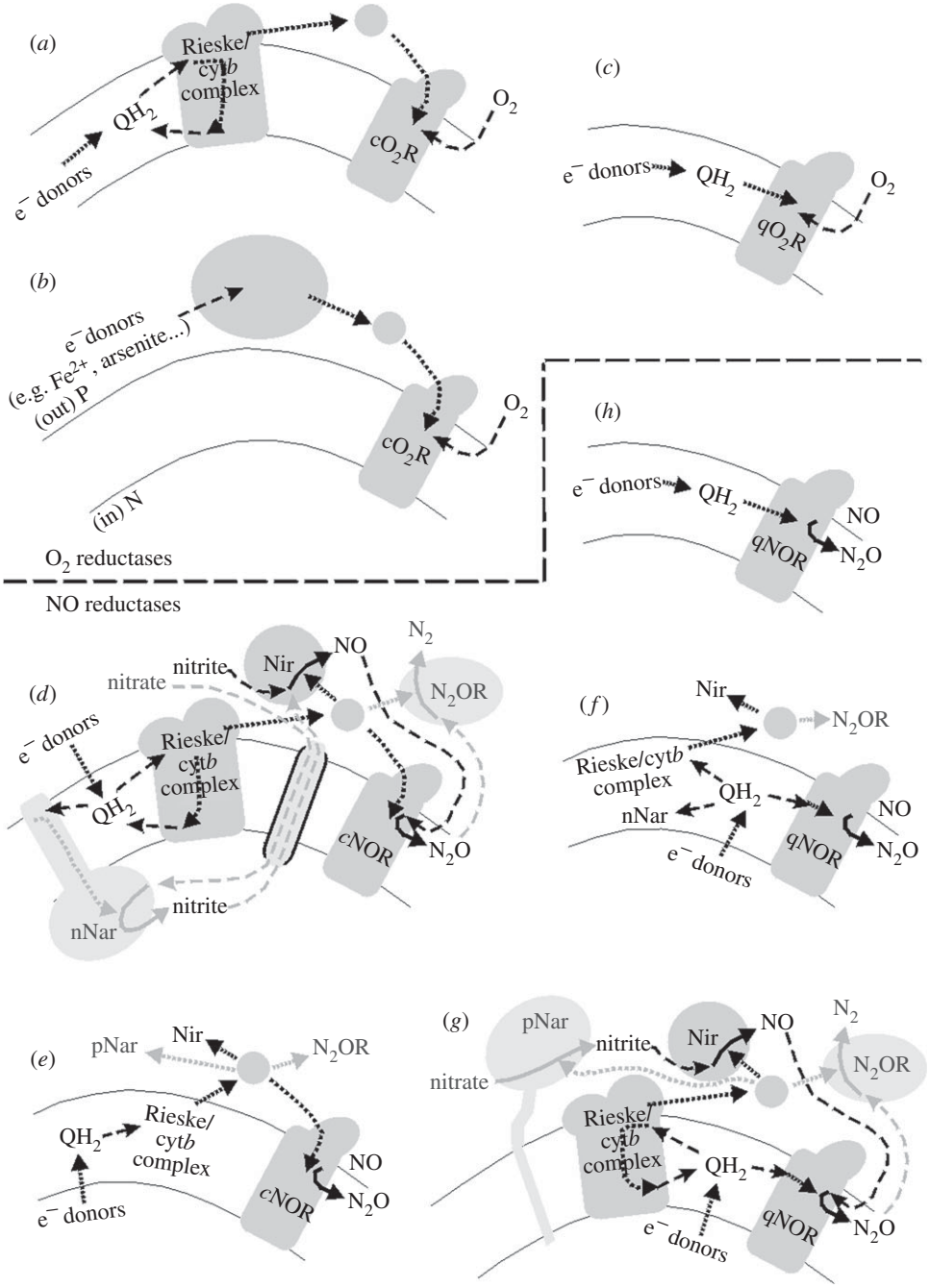

Figure 1. (*a–c*) Schematic of how O_2Rs and (*d–g*) NORs may be functionally integrated in (aerobic and anaerobic) respiratory chains. Nar, Nir and N_2OR stand for nitrate, nitrite and nitrous oxide reductases, respectively, QH_2 denotes quinols. The 'p-' and 'n-' prefixes denote Nar enzymes the catalytic sites of which are located on the positive (typically outside) and negative (typically inside) faces of the chemiosmotic membrane, respectively. Dotted arrows indicate electron transfer, dashed ones stand for transport of substrates and continuous arrows denote substrate conversions. Lighter shade enzymes and arrows indicate reactions and electron transfer steps, respectively, that may be absent in a given scheme (e.g. nitrate- and N_2O reduction are not part of all chains). Electron donors can be located on either side of the membrane according to their chemical nature and specific chain but these different locations are not indicated in the scheme for reasons of simplicity.

Experimentally demonstrated as well as likely (from the coexistence of genes coding for the corresponding enzymes in respective genomes) types of interactions between O_2/NO reductases and further bioenergetic enzymes are schematically depicted in figure 1. The situation is relatively simple for the case of the O_2Rs (top section of figure 1). The 'classic' system encountered in mitochondria and selected proteobacteria is shown in figure 1*a* and corresponds to a cO_2R which receives reducing equivalents *via* a periplasmic soluble electron carrier protein from the Rieske/cytochrome *b* complex (the bc_1 complex in mitochondria and α-/β-/γ-proteobacteria) which itself oxidizes quinols in the famous Q-cycle reaction [20–22]. In addition to the traditional scheme of figure 1*a*, several examples have been reported in prokaryotes where electrons arising from reducing nutrient substrates do not pass through the Rieske/cytochrome *b* complex but feed directly into the soluble-carrier donating electrons to the O_2R as illustrated in figure 1*b* [23–25]. In all cases we are aware of, the reason for bypassing the Rieske/cytochrome *b* complex and donating directly to the soluble electron carrier protein is clearly thermodynamic, that is, the corresponding substrates are not reducing enough to afford the free energy required for the proton-motive-force- (pmf-) generating detour through the Q-cycle. For the Q-cycle to operate, the respective substrates need to be able to reduce the quinone pool, which is electrochemically challenging for substrates such as for example Fe^{2+}.

The right-hand side column of figure 1 shows electron transfer schemes involving quinol-oxidizing O_2Rs and NORs. Figure 1*c* illustrates the scheme as for example observed in the well-studied qO_2R, the so-called *bo* oxidase, of *Escherichia* (*E.*) *coli* [26].

The extreme simplicity of this qO_2R scheme is made possible by the fact that the enzyme's substrate, O_2, is an environmental one, i.e. dissolved O_2 is more or less plentiful in the environments the respective organisms grow in. This is not the case for NO. Except for the very rare cases when NO is generated in chemical warfare between species, free-living microorganisms do not encounter NO in the environment. However, NO occurs as an intermediate in the chain of reduction steps converting nitrate and/or nitrite, which are abundant in specific habitats (and increasingly so due to anthropogenic pollution of the planet), to either N_2O or all the way to N_2. The occurrence of an NOR therefore in the vast majority of cases does not make bioenergetic sense except in the light of its functional integration into a chain of further energy conserving enzymes. Such a chain is nicely illustrated by the intensely studied denitrifying system of the α-proteobacterium *Paracoccus* (*P.*) *denitrificans* [27,28] as depicted in figure 1*d*. This system features a cNOR drawing the reducing equivalents required for catalysis from a pool of soluble *c*-type cytochromes and getting its substrate NO produced *in situ* through the action of a periplasmic nitrite reductase (Nir) for which two completely unrelated versions exist, that is, the Cu-containing 'Cu-Nir' [29] and the sirohaem-containing 'cd_1-type' [30] enzyme. Both types of Nir, just like cNOR, drain electrons from the pool of soluble cytochromes. Electrons are furnished to this cytochrome pool by the Rieske/cytochrome *b* complex and ultimately by environmental reducing substrates replenishing the pool of quinols. A number of prokaryotes content themselves with this minimal scheme comprising Nir, cNOR and the Rieske/cytochrome *b* complex, whereas others complement this minimal chain either on its oxidizing or its reducing end or on both by adding nitrate reductase (Nar) producing nitrite and/or N_2O reductase (N_2OR) which further reduces the dissolved gas N_2O produced by cNOR to N_2. N_2OR again taps into the pool of periplasmic electron carriers, whereas the Nar of most prokaryotes is directly reduced by the quinone pool. The catalytic site of such Nars lies in the cytoplasm implying that the environmental substrate nitrate must be transported across the cytoplasmic membrane and the reaction product, nitrite, needs to be exported to be available as substrate for the periplasmic Nir. These transport events are mostly carried out by ATP-consuming nitrate/nitrite antiporters as indicated in figure 1*d*.

The scheme of figure 1*d* was long considered *the* way a denitrifying chain is organized. However, in 2007 it became clear that members of the phylum Haloarchaea arranged their nitrate reducing bioenergetic system quite differently [31–33]. In fact, in several archaeal species Nar was found to be a periplasmic enzyme lacking the transmembrane (TM) electron wire but possibly being tethered to the membrane [34]. Reflecting their membrane-sidedness and following the nomenclature proposed by Martinez-Espinosa *et al*. [33], the two differing Nars are in figure 1 distinguished as nNar (*Paracoccus* case) and pNar (haloarchaeal scheme). The major functional difference between these two groups thus consists of the fact that pNar receives its electrons from the pool of soluble carriers (which in the case of the haloarchaea likely is a cupredoxin) rather than from the quinone pool. Of course, owing to the periplasmic position of pNar, TM-transport of nitrate and nitrite is not required.

Several articles have recently called into question whether results obtained on haloarchaea may be straightforwardly interpreted as representative for the archaeal domain. Haloarchaea have apparently in the distant evolutionary past undergone massive import of genes from donors in the domain of the Bacteria [35] and the majority of enzymes functioning in bioenergetics indeed show bacterial and not archaeal origins [36]. One might therefore suspect that the peculiar arrangement of enzymes in haloarchaeal denitrification is an exotic phenomenon due to the reconstitution of respiratory electron transport from a multiplicity of sources. Some of us have recently examined the species distribution and phylogeny of pNar and nNar (B Schoepp-Cothenet, F Baymann, A Magalon, W Nitschke 2014, unpublished data) and the results obtained clearly indicate that this is not the case. Archaeal nitrate reduction is based almost exclusively on pNar-type enzymes and the full chains likely resemble that shown in figure 1*g*.

Apart from the membrane-sidedness of Nar, the scheme in figure 1*g* furthermore differs from the above detailed scheme of figure 1*d* by the presence of qNOR rather than of cNOR. This representation is based on our inventory of denitrification-related genes in sequenced genomes of Archaea and Bacteria. The overwhelming majority of species containing pNar in fact show a coexistence of qNOR- and the absence of cNOR-encoding genes in their genomes. We therefore tend to assume that using pNar entails a propensity for employing qNOR and we are tempted to attribute this fact to thermodynamic constraints, i.e. the necessity to avoid excessive oxidations states of the pool of soluble donors as will be discussed below (§10.1.). Still, the very rare exceptions to this rule in the genomic inventory leave the possibility that the pNar/qNOR correlation may not be fully stringent. We therefore have also included (simplified) hypothetical schemes associating nNar with qNOR and pNar with cNOR in figure 1*f*,*e*, respectively. To the best of our knowledge, no such chain has so far been characterized.

Figure 1*h* shows a scheme analogous to that depicted for qO_2Rs in figure 1*c*, i.e. devoid of an internal source for NO and only containing an unspecified system of enzymes reducing the quinone pool. According to all we have said above, such a system should not occur in nature due to the scarcity of environmental NO. Nevertheless, our inventory of gene distributions suggests that a number of cyanobacteria clearly do possess qNOR but no other enzyme of the denitrifying pathway apart from the Rieske/cytochrome *b* complex. It remains to be sorted out whether these enzymes serve as defence mechanisms against competitor-generated NO or whether they fulfil very different roles along the lines of what has recently been proposed [37] for two of the three qNOR gene-products found in the denitrifying methanotroph *Methylomirabilis oxyfera* (see §9).

3. Evolution has its own way to class

Figure 1 shows that there are four types of O_2/NO reductases if only the basic functional parameters of substrate type and nature of the electron donor are considered, that is, cNOR, qNOR, cO_2R and qO_2R. A bioenergetic engineer would likely have developed four dedicated and specialized enzymes optimized for these four basic tasks. Since, as François Jacob

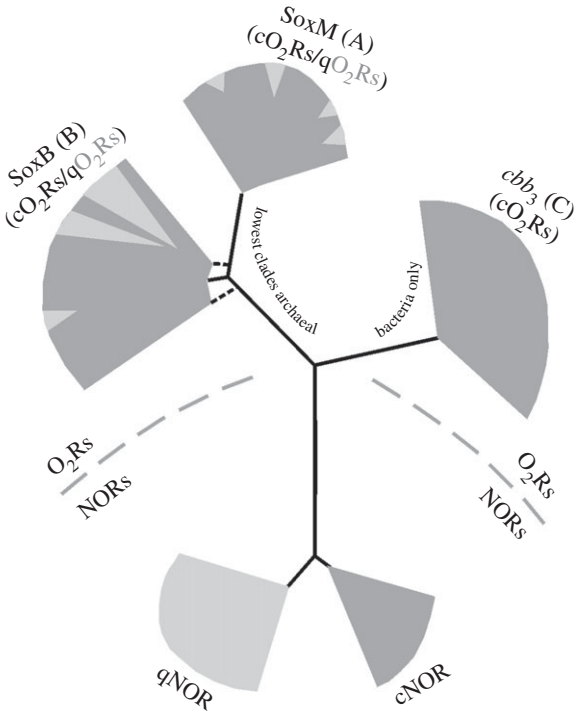

Figure 2. General appearance of the composite phylogenetic tree encompassing all subgroups of the superfamily. SoxM (A), SoxB (B) and *cbb*$_3$ (C) are O_2 reductases, whereas qNOR and cNOR stand for quinol- and soluble-carrier-oxidizing NORs as defined in [3,8]. Dark shadings denote enzymes that oxidize soluble electron donors and lighter shadings stand for quinol-oxidizing representatives. The dashed lines connecting the SoxB (B) group to the main stem of the tree are meant to indicate that in most recent trees, SoxB (B) tends to split into two distinct clades (e.g. [15]). The figure represents the general outline of the enzyme superfamily's phylogeny [8,10,14,15].

famously said, evolution is a tinkerer, the situation we actually encounter in biology is substantially more complicated. However, as noted by Saraste and co-workers [3] 20 years ago, all types of respiratory O_2 reductases share significant amino acid sequence similarity among each other and with all members of respiratory NORs. This enables the derivation of a classification based on sequence and hence genealogical proximity based on the methods of molecular phylogeny. Such phylogenies reconstructed from the sequences of the membrane-integral catalytic subunits from all these enzymes yield a tree featuring at least five relatively well-defined clusters (schematically in figure 2). Three of these clusters contain O_2 reductases commonly referred to as either A-, B- and C-type [8] or SoxM-, SoxB- and *cbb*$_3$-type [6] enzymes. The older nomenclature proposed by Saraste *et al*. [6] is now widely considered obsolete and has been replaced by the alphabetical scheme containing three major groups as introduced by Pereira *et al*. [8]. Furthermore, a classification scheme with more subdivisions which breaks down the three major clades depicted in figure 2 into a plethora of subgroups has furthermore been proposed by Hemp & Gennis [9]. The additional dashed lines connecting SoxB (B)-type enzymes to the main tree are meant to indicate the fact that the most recent published phylogenies [14,15] including ours (see below, figure 7*a*) are in favour of two distinct clades rather than a single SoxB (B) clade.

We admit that the historical character of the term 'Sox', referring to '*Sulfolobus* oxidase', may make this nomenclature sound old-fashioned as compared with the seemingly more sober and thus scientifically more appropriate alphabetical nomenclature. However, as we will argue below, the alphabetical scheme may not be so appropriate after all. The A/B/C-labelling of the three major clades in figure 2 in fact conveys the idea that all these three clades are on the same cladistic level. We consider this as evolutionarily misleading. Several non-phylogenetic properties to be discussed below in fact suggest that A and B are sister-groups and that this A + B-supergroup has diverged from a common ancestor with the C-group. Such an evolutionary proximity is better rendered by the closely related terms SoxM and SoxB as opposed to the phonetically (just as structurally) very different *cbb*$_3$-type enzyme. We therefore have a strong preference for the older nomenclature which in our minds reflects evolutionary descent more fittingly. Nevertheless, for the sake of minimizing confusion, we will indicate both labelling schemes when referring to the different types of O_2 reductases throughout this article.

Intriguingly, the three different clades of O_2Rs do not reflect the classification scheme suggested by function (figure 1). SoxM (A)- and SoxB (B)-type oxidases contain both cO_2R (darker shading in figure 2) and qO_2R (lighter shading) in a strongly mixed manner and only the *cbb*$_3$ (C)-type O_2R so far appears to shun quinols as electron donors and stay true to their soluble redox partners. The so far published phylogenies [9,11,14,15] additionally suggest the presence of two major groups of NORs which are generally identified with qNORs and cNORs (figure 2). As we shall see below (§6.1.), the tree of NOR presently goes through a growth spurt. The wealth of structural information now available on NORs allows a more reliable interpretation of sequence idiosyncrasies in the multiple subgroups of both NOR clades yielding a better idea of the functional conservations and variations within these two clades as detailed in §5.

4. The riddle of the phylogenetic tree

The necessary condition in molecular phylogeny for inferring that a given enzyme family may have been around prior to the divergence of the prokaryotes into Archaea and Bacteria is that the phylogenetic trees reconstructed from its constituent proteins (or genes encoding these proteins) also diverge at their base into archaeal and bacterial subtrees. Owing to the possibility of horizontal gene transfer even between the domains of Archaea and Bacteria, a few branches from the opposite domain may well be present in these subtrees but their root, defining the divergence point, should separate essentially archaeal from essentially bacterial subtrees.

In addition to NORs, three distinct groups of O_2Rs are seen to emanate from the common 'stem' of the tree in figure 2. None of these groups of O_2Rs fulfil the above-mentioned criterion. *cbb*$_3$ (C)-type enzymes have (so far) exclusively been found in Bacteria. Of course, absence of evidence for archaeal representatives is not evidence for their absence and conclusions drawn today may need to be revised tomorrow if archaeal *cbb*$_3$ (C)-type enzymes should show up in newly sequenced genomes. However, as mentioned, archaeal representatives by themselves would not yet require overturning of conclusions; to do so, they also have to diverge basally from the present tree. As the body of data and their resulting tree stand today, one therefore has to infer that *cbb*$_3$ (C)-type O_2Rs originated in Bacteria and were inherited mainly vertically within the bacterial domain together with a few

laterally transferred representatives [10]. Both in the SoxB (B) and the SoxM (A) clade as they appear in the composite tree of NORs and O_2Rs (figure 2), the lowest branching clusters are exclusively archaeal as already noted previously [11] and the pertinence of this observation has only strengthened with the multitude of genomes sequenced during recent years. The topology of the composite tree taken at face value therefore indicates that SoxB (B) and SoxM (A) originated in Archaea and were only later laterally transferred into the bacterial domain.

The respective origins of cbb_3 (C) and SoxB/M (A/B) in Bacteria and Archaea, however, requires that there was an ancestor to both of them in the LUCA, a conclusion which is further corroborated by the fact that the common root of all O_2Rs connects up to NORs which also can be argued to have been present in the LUCA [7,11] (see §6.1). The substantial structural differences between cbb_3 (C)-type O_2Rs on one side and SoxB/M (A/B) enzymes on the other (see §5) raises the questions of not only how their common ancestor may have looked like but also what it actually did. Of course, the first guess would be that the common ancestor of two O_2Rs was an O_2R. This, however, clashes with the prevalent palaeogeochemical notion of an essentially oxygen-free early planet (see §7) as already remarked in the early days of studies on the evolution of the superfamily [5]. The reconciliation of phylogeny and palaeogeochemistry was therefore proposed to come from the very differences between cbb_3 (C)- and SoxB/M (B/A)-type oxidases [11]. These two groups in fact differ in the sequence position of a catalytically crucial residue, a redox active tyrosine (for a review on the functional mechanism of O_2Rs, see [38]). This residue is essential for the four-electron reduction and protonation of O_2 but not for the two-electron reaction chemistry of NO and correspondingly is absent in NORs. The strict sequence dichotomy between cbb_3 (C)- and SoxM/A (A/B)-type enzymes with respect to this residue crucially important for O_2 reduction led to the proposal that the ancestor in LUCA of cbb_3 (C)- and SoxB/M (A/B)-type O_2Rs actually was not an O_2R but a NOR and that the evolutionary transition to O_2Rs occurred twice independently in Bacteria and Archaea when O_2-levels became sufficiently high [11]. This picture of an O_2-using enzyme emerging from a precursor operating in an anaerobic environment is generally in line with results from large-scale genome comparisons of protein folds involved in aerobic and anaerobic metabolism [39,40].

At this point, we have to admit that the way of presenting the global phylogeny of the superfamily in figure 2 is shared by several [4,7–9,15] but not all colleagues [12,13]. We still do hold, however, that all phylogenies reconstructed on the basis of the total set of NORs and O_2Rs yield a tree globally similar to that shown in figure 2. A route differing from that described above to resolve the intrinsic complications of the tree was taken by Brochier-Armanet and co-workers [12,13]. These authors considered that the apparent inconsistencies of the tree are due to basic tree reconstruction problems, that is, that the phylogenetic tree does not reflect evolutionary reality. As a consequence, they have studied the individual clades, i.e. SoxM (A), SoxB (B), cbb_3 (C), qNOR and cNOR, separately and derive, based on phylogenomic arguments, which of these groups should be considered as the most ancestral one and which ones as derived. A key observation used by these authors was that by placing the root differently from what is suggested by the phylogenetic trees of the entire superfamily, the SoxM clade can be rendered 'bipartite' falling into an archaeal and a bacterial subclade. These lines of reasoning lead Brochier-Armanet *et al*. to propose SoxM (A), the enzyme most widely distributed in extant organisms, as the most ancestral O_2R present already in the LUCA, whereas all other groups of O_2Rs and NORs would be later evolutionary emanations from this ancestral SoxM (A)-type enzyme. This scenario was subsequently adopted by the Gennis group and is hence used by these colleagues as a basis for interpreting structural and functional results [1].

It is noteworthy that the scenario put forward by Gribaldo *et al*. [13] results in a sequence of events diametrically opposite to what is proposed above. A low-affinity O_2R would in this scenario have originated in the Late Hadean or the Early Archaean, at any rate in the LUCA, and hence long before photosynthetic production of O_2. This obviously requires a non-photosynthetic source of molecular oxygen at least in certain habitats on the early Earth [13]. High-affinity SoxB (B)-type O_2Rs would have appeared when organisms ventured out of these habitats rich in O_2 into less oxygenated environments [1].

The following sections will compare available three-dimensional structures of enzymes from the five groups of O_2Rs/NORs in an effort to better understand the evolutionary trajectories linking these groups together.

5. Gleaning nature versus nurture from the (X-ray) family photograph

5.1. A view from the distance

Grouped around the common catalytic core in figure 3, we show five three-dimensional structures representing the phylogenetically defined clades. The choice of representative for qNOR/cNOR-, cbb_3 (C)- and SoxB (B)-type O_2Rs is a no-brainer since only one structure each is presently available. By contrast, for SoxM (A)-type enzymes, several structures from taxonomically diverse species have been solved. We consider the caa_3-type O_2R from *Thermus thermophilus* as most closely corresponding to the archetypal SoxM (A) enzymes for the following reasons: (i) on phylogenetic trees reconstructed from sequences of the catalytic subunit, this enzyme occupies the earliest diverging branch of all O_2Rs for which three-dimensional structures are presently in the databases. The phylogenetic tree of SoxM (A) does feature branches diverging even earlier but for the time being, this enzyme is the best we have. (ii) The *Thermus* caa_3 enzyme contains the cytochrome subunit fused to the C-terminal end of Subunit II (SUII) (see below). As discussed below and substantiated in a separate research article (R van Lis, W Nitschke, A-L Ducluzeau 2014, unpublished data), the SUII–cytochrome fusion likely is an ancestral trait of the SoxM (A) clade.

While the general outlines of qNOR and cNOR appear to strongly resemble each other when observed at the scale of figure 3, they nevertheless are set apart by the fact that the peripheral, solvent-exposed subunit in cNOR is a *c*-type cytochrome, whereas the structurally corresponding domain of qNOR does not carry redox cofactors. The three clades of O_2R, however, reveal themselves as substantially diverging already with respect to global subunit composition. The cbb_3 (C)-type O_2R contains only haem *c*-carrying extrinsic subunits. The cytochrome subunit of the cbb_3 (C) enzyme which is closest to the catalytic subunit (so-called 'CcoO', dark red in figure 3) is conserved throughout the entire cbb_3 (C) clade [10], whereas the

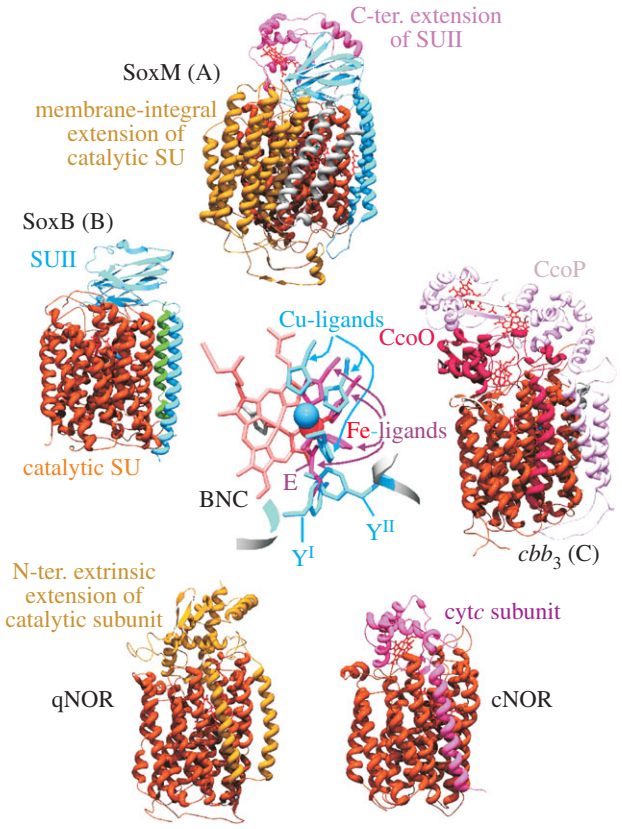

Figure 3. Ribbon-style representation of the three-dimensional structures of representatives from all five major groups of the NOR/O_2R superfamily. The catalytic subunit common to all enzymes is drawn in orange and other subunits and domains are coloured as indicated in the figure. The general layout of the catalytic BNC for O_2Rs and NORs is shown in the centre of the figure. The ligands to Cu_B of O_2Rs as well as the two distinct redox active tyrosines Y^I (SoxM/B) and Y^{II} (*cbb*$_3$) are shown in blue, whereas those binding Fe_B are depicted in dark red. Cu_B and Fe_B are represented as blue and red spheres, respectively. Pdb-entry numbers: 2YEV (SoxM (A) *caa*$_3$), 1EHK (SoxB (B) *ba*$_3$), 3MK7 (*cbb*$_3$ (C)), 3AYG (qNOR), 3OOR (cNOR). Structures in this figure as well as in all other figures were drawn using DeepView (www.expasy.org/spdbv/) [41].

presence of the 'CcoP' dihaem protein (mauve) is restricted to later-branching representatives where it has apparently replaced the evolutionarily more ancestral monohaemic CcoR cytochrome [10]. In striking contrast to both NORs and *cbb*$_3$ (C-type) cO$_2$Rs, the SoxM (A) and SoxB (B) enzymes both harbour the structurally conserved extrinsic SUII featuring a cupredoxin fold (cyan). SoxM (A) distinguishes itself from SoxB (B) by the presence of additional protein domains or subunits: (a) the membrane-integral subunits III and IV (orange) which in many representatives of SoxM (A)-type O_2Rs are fused to the C-terminal end of the catalytic subunit and (b) a haem-carrying C-terminal extension (violet) of SUII corresponding to a class I cytochrome. Whereas genes coding for SUIII/IV-related polypeptides are conserved in almost all SoxM (A) gene clusters, the cytochrome extension of SUII is not present throughout the clade. However, all SUIIs from the SoxM (A) clade carry C-terminal extensions of variable lengths in striking contrast to SUIIs from SoxB (B)-type enzymes which all stick to the standard cupredoxin motif as inferred from the multiple sequence alignments of SUIIs from SoxB (B) and SoxM (A).

The view from the distance suggests four different types of extrinsic subunits (disregarding for the moment the additional cytochrome domain of *caa*$_3$-type cO$_2$Rs), i.e. the cupredoxins of SoxM (A)- and SoxB (B)-type enzymes, the CcoO cytochrome of *cbb*$_3$-type cO$_2$Rs, the monohaem *c*-type cytochrome subunit of cNOR and the so far unspecified solvent-exposed part of qNOR which is strictly speaking not a subunit but corresponds to an N-terminal extension of the conserved catalytic subunit. In the following, we will look at the extrinsic polypeptides in more detail.

5.2 The extrinsic subunits, small but rich in evolutionary meaning

Figure 4 shows a close-up comparison of the monohaem subunits of *cbb*$_3$ (C)-type cO$_2$R and cNOR with the extrinsic domain of qNOR and the cytochrome domain of the *caa*$_3$-type cO$_2$R from *Thermus*, the latter being intended to serve as an example of a standard class I cytochrome. Strikingly, the extrinsic polypeptide chains of cNOR and of qNOR show a very similar fold apart from a lengthy insertion in qNOR (marked in grey in figure 4). The root-mean-square (RMS) deviation between these two polypeptides is by far the lowest of all diades of figure 4, and the number of atoms of the carbon backbone which can be well-aligned is the highest (see table insert in figure 4). This obvious structural similarity extends to sequence homologies already noted more than a decade ago [42]. Intriguingly, the gene coding for the *c*-type cytochrome is located directly upstream of the gene which encodes the catalytic subunit in all cNORs, suggesting a facile gene fusion or gene split event transforming cNOR into qNOR or *vice versa*. The only blemish of the gene fusion/split scenario lies in the presence of an additional TM helix linking the P-side-exposed domain to what would be the N-terminus of cNOR which is located on the N-side of the membrane. It might seem suspicious from an evolutionary perspective that in structural overlays of all members of the NOR/O_2R superfamily, this additional helix superimposes well with corresponding helices in all other clades except cNOR. Is the presence of this helix therefore an ancestral trait of the superfamily? We consider this highly unlikely since the polarity of the qNOR TM helix actually is opposite to that of all other structurally related TM helices. The qNOR TM helix runs from the P- to the N-side, whereas in all other cases the structurally closely related helices go the other way round. This strongly suggests that the helix was either captured in qNORs or lost in cNORs and that its structural proximity to those of the O_2Rs is fortuitous or due to convergent evolution driven by still poorly understood structural constraints.

All this leaves little doubt that the *c*-type cytochrome subunit of cNOR and the N-terminal peripheral domain of qNOR are evolutionarily closely related as opposed to, for example, the cupredoxins of SoxM(A)/SoxB(B) and the CcoO cytochrome of *cbb*$_3$ (C)-type enzymes which clearly have independent evolutionary origins. The peripheral subunit/domain of cNOR and qNOR both feature the canonical fold of class I-cytochromes with three α-helices wrapping around the haem moiety in a characteristic manner (see for example the *c*-haem domain of *caa*$_3$-type cO$_2$R in figure 4 which is a typical member of the class I family of cytochromes).

The class I fold admittedly is also present in the CcoO protein (figure 4) from *cbb*$_3$ (C)-type O_2Rs. However, the presence of long insertions, undetectable sequence homology (apart from the haem-binding CXXCH motif) and the very poor structural fit (table in figure 4) suggest that the CcoO

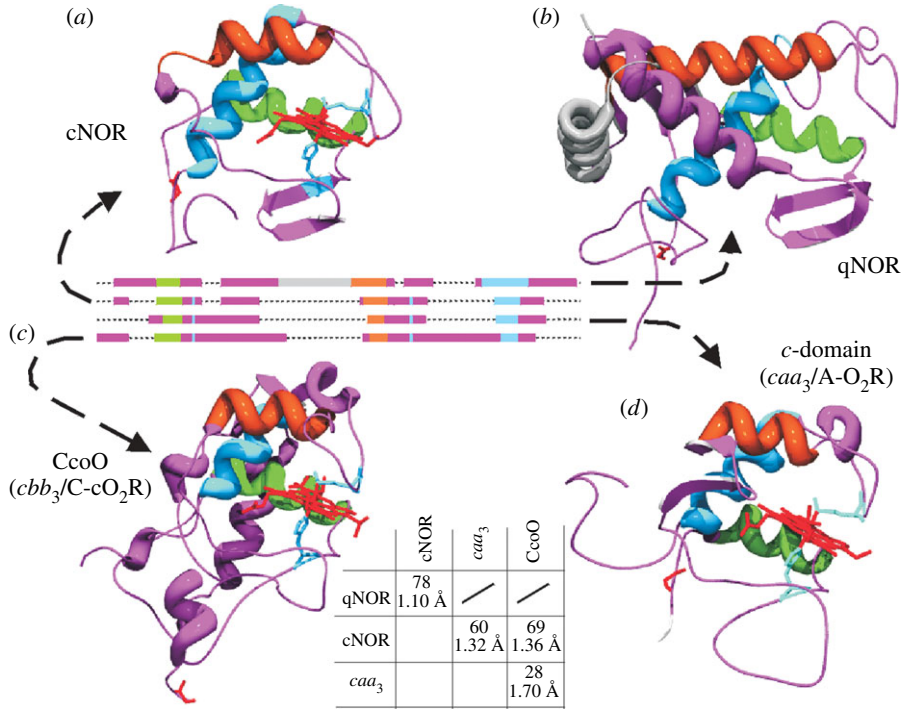

Figure 4. Comparison of three-dimensional structures of the membrane-extrinsic parts of the cytochrome subunit in cNOR (*a*), the N-terminal domain of qNOR (*b*), the CcoO subunit from cbb_3 (C)-type cO_2Rs (*c*) and the cytochrome *c*-domain of SUII in caa_3-type cO_2Rs (*d*). pdb-entry numbers are as indicated in the legend to figure 3. The canonical triade of α-helices nesting the haem group in class I cytochromes is shown in green, orange and blue. The remaining bulk of protein is depicted in violet except from the major extension specific to qNOR which is shown in grey. Axial ligands to the haem are represented in blue. A sequence representation of the structural alignment is shown in the middle of the figure using the same colour code as for the three-dimensional structures. Inset: table representing root-mean-square (RMS) deviations for structurally aligned α-carbons (the number of well-aligned backbone carbons is indicated above the RMS values) between pairs of structures. Structure alignments and RMS values were calculated using DeepView [41].

subunit represents an independent recruitment of a class I *c*-type cytochrome at the base of the cbb_3 (C) clade.

In contrast to these peripheral polypeptides derived from the class I-fold but featuring extensive idiosyncrasies, the extrinsic SUIIs of SoxB (B)- and SoxM (A)-type O_2Rs deviate only marginally from the canonical fold of cupredoxins. Figure 5 shows a structure comparison of these subunits from diverse Sox-type O_2Rs for which three-dimensional structures are available to the cupredoxin fold as typified by amicyanin or the C-terminal domain of N_2OR. This comparison indicates that SUII from SoxB (B)-type enzymes conserves the cupredoxin structural motif particularly well apart from the additional presence of the N-terminal TM helix. Judging from sequence comparisons, this is true for all SoxB (B)-type O_2Rs. As already mentioned above, almost all SoxM (A) enzymes, by contrast, contain C-terminal extensions of variable lengths, the longest corresponding to the fully fledged class I cytochrome of the *Thermus* caa_3-type O_2R and the likes as depicted in figure 4 (or even a dimer of class I cytochromes as in sulfate reducing δ-proteobacteria [43]). It is noteworthy that despite their conserved cupredoxin fold, not all SUIIs are in fact copper proteins. Absence of the copper-ligating residues does not alter the global fold of the protein but eliminates the di-metal Cu_A-site. The enzymes thus devoid of the Cu_A redox centre have lost the ability to oxidize soluble electron carrier proteins and in general retrieve their reducing equivalents from the quinone pool, i.e. they are qO_2Rs. As was shown more than two decades ago in a beautiful site-directed mutagenesis study, the Cu_A-binding site can be reintroduced into the qO_2R from *E. coli* transforming the enzyme back to a cO_2R [44]. This finding together with the fact that qO_2R-clusters in phylogenetic trees of the enzyme invariably emanate from within cO_2R clades (figure 2) rather convincingly demonstrates that qO_2Rs are evolutionarily derived from cO_2R.

The general picture arising from the structural features of the extrinsic subunits thus is that of only three evolutionarily independent motifs: (i) the NOR-architecture common to both qNOR and cNOR, (ii) the cbb_3 (C)-type layout with its conserved CcoO *c*-type cytochrome and (iii) the cupredoxin motif common to SoxB (B)- and SoxM (A)-type cO_2Rs or qO_2Rs. In this scheme, the SoxM (A)-type enzyme appears as having emerged from the SoxB (B) type structural motif through the addition of protein mass both at the membrane-integral and at the solvent-exposed periphery of the enzyme.

5.3. The proton channels: NOR structures reopen the case

SoxM (A)-type O_2Rs were recognized a long time ago (both *via* structures and through site-directed mutagenesis studies) to feature two distinct channels conducting protons from the N-side of the membrane to the catalytic BNC. Referring to the residues crucial for proton entry from the N-side into the mitochondrial enzyme, a lysine and a glutamate, these two proton channels have been dubbed the 'K'- and the 'D'-channel (figure 6). The three-dimensional structure of the SoxB (B)-type O_2R from *Thermus* [45] has shown that the D-channel is absent in this enzyme, and multiple alignments indicate that this is true throughout the SoxB (B) subfamily [16]. The K-channel, by contrast, is relatively well conserved [45] (figure 6). Although slightly less obvious in sequence alignments, the

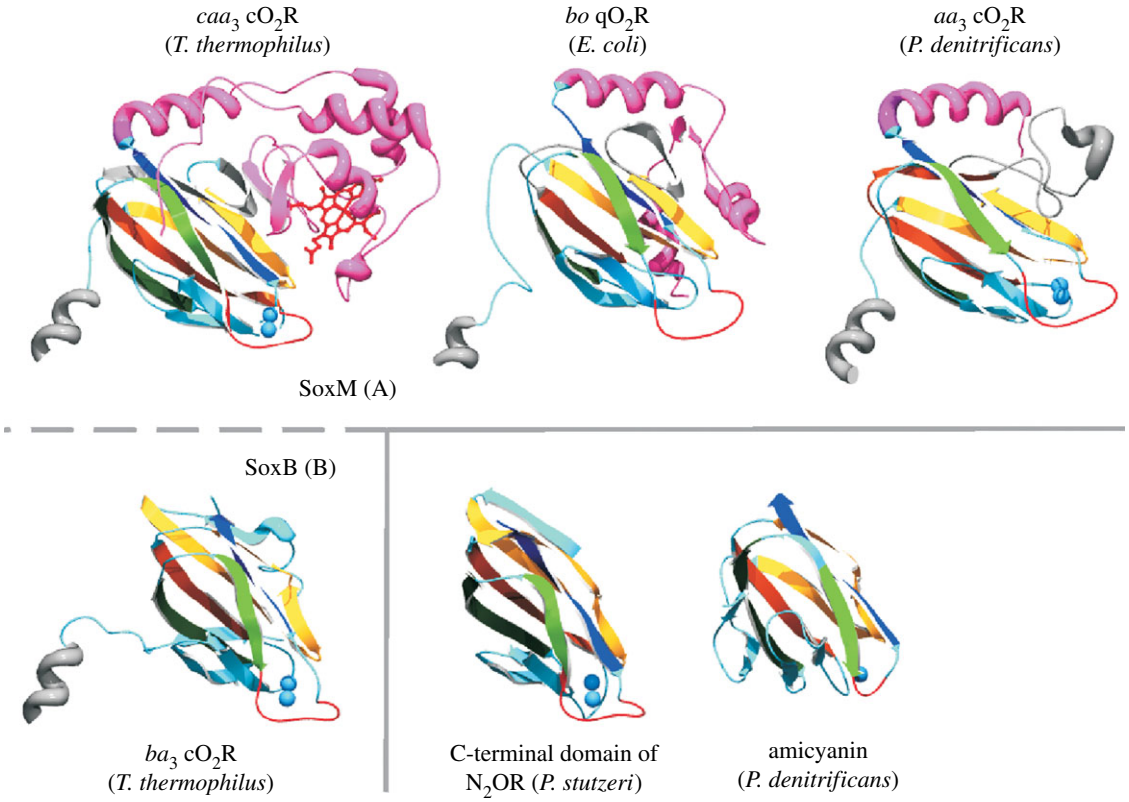

Figure 5. Comparison of the three-dimensional structures of SUIIs from three different SoxM (A)-type enzymes (pdb-entries: 2YEV for caa_3, 1FFT for bo_3 and 1QLE for aa_3) and the unique SoxB (B) representative from *T. thermophilus* (1EHK) to those of the cupredoxin-type domain (containing a dinuclear Cu_A centre) of N_2OR (3SBQ) and the soluble electron carrier protein amicyanin (2OVO). For the sake of more facile comparison, the consecutive β-strands of the cupredoxin fold have been coloured identically in all structures. The pink protein mass shown for SoxM (A)-type SUIIs are the C-terminal extensions exceeding the canonical cupredoxin fold.

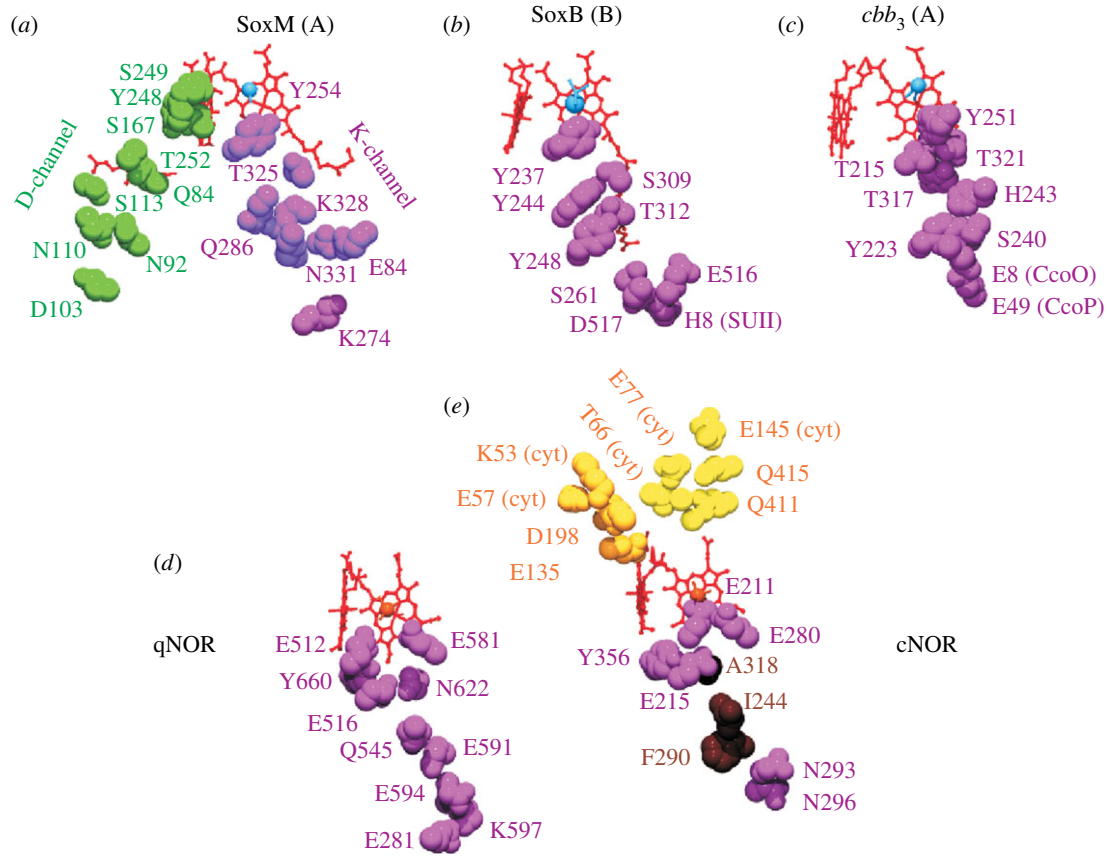

Figure 6. Structural comparison of demonstrated and proposed proton channels towards the BNC in O_2Rs (*a*–*c*) and NORs (*d*,*e*). D-channel residues are denoted in green, whereas the K-channel and structural analogues thereof are shown in violet. The two hydrophobic and bulky residues interrupting the putative K-channel of cNOR are shown in brown, and the replacement channels transporting protons from the P-side towards the BNC are marked in lighter and darker shades of yellow. Residue numbering is according to the respective pdb-files as indicated in figure 3.

K-channel is also structurally conserved in cbb_3 (C)-type enzymes, while the D-channel is again absent in this class of O_2Rs (figure 6) [46]. The absence of the D-channel has been functionally correlated with a reduced proton pumping stoichiometry [1,2].

NORs, by contrast, have throughout the respective literature been considered to not pump protons but to receive the H^+ required for the reduction of NO from the P-side of the membrane, that is, to not be electrogenic. Such a scenario has been vindicated by the structure of cNOR which does not feature a continuous K-channel but which contains two likely proton conducting pathways from the P-side to the catalytic BNC [47,48] (figure 6). Surprisingly, however, a K-channel equivalent can be detected [48,49] in the structure of qNOR! It is noteworthy that qNORs have been much less extensively studied with respect to proton translocation, and the structural data are presently stimulating a reassessment of electrogenicity in qNORs. First results indeed indicate substantial differences in proton-uptake characteristics between cNORs from proteobacteria and the qNOR from *Geobacillus* (*G.*) *stearothermophilus* [50]. Even more astonishing but also extremely intriguing from the evolutionary point of view is the observation that several of the residues forming the K-channel equivalent in qNOR are also found in cNOR (figure 6). The main difference between qNOR and cNOR K-channels in fact lies in the exchange of two potentially proton conducting residues in the middle of the qNOR channel by strongly hydrophobic and bulky residues (isoleucine and phenylalanine) in cNOR. As noted in the articles comparing the two NOR structures [48,51], this looks as if an existing K-channel in cNOR was blocked by shoving an insulating cork into the proton conduit.

The picture emerging from figure 6 therefore is that of an ancestral K-channel present in the entire NOR/O_2R superfamily but shut down in cNOR and amended by the D-channel in SoxM (A)-type O_2Rs. Stimulated by the possibility that a functional K-channel might have been present at the root of the cNOR clade, we have inspected the multiple sequence alignment arising from our resampling of NOR-related gene clusters in available genomes (§6.1). The proton conducting residues seen in the qNOR structure but being exchanged against hydrophobic amino acids in the three-dimensional structure of cNOR from *Pseudomonas* (*P.*) *aeruginosa* in fact turn out to be conserved in more than half of all cNOR subclades. Most intriguingly, among the representatives which putatively have this K-channel is the recently characterized cNOR from *T. thermophilus* [52]. Site-specific mutagenesis data on this enzyme targeting the channels from the P-side as seen in the *P. aeruginosa* cNOR structure turned out to be inconclusive [52]. This finding would indeed be expected if the protons were delivered from the N-side of the membrane *via* a K-channel in the *T. thermophilus* cNOR. We take all this to indicate that a K-channel may indeed have been present at the base of the cNOR clade but was shut down in selected clades (for a discussion of possible reasons, see §10.1). We therefore consider it highly likely that all NORs (and by extension the whole superfamily) set out with a fully functional K-channel.

5.4. Catalysis: why E? Y copper?

The scheme in the centre of figure 3 summarizes the structural layout of NOR/O_2R's BNC, buried within the membrane-integral catalytic subunit (orange in figure 3). The BNC consists of a five-coordinate haem and a non-haem metal centre, and the substrate-binding site is sandwiched in between the haem-iron and the second metal centre.

The catalytic reactions of NOR and O_2R are actually quite dissimilar. NOR catalyses the two-electron reduction of two NO molecules and two protons to yield a single N_2O (a dissolved gas) and one molecule of water. The fact that this reaction thus is bimolecular in NO has implications for the strong dependence on the concentration of dissolved NO, as a driving force in NOR as will be detailed in §10.1. O_2 reduction, by contrast, is monomolecular requiring four electrons and four protons to yield two water molecules. As a consequence of these reaction dissimilarities, NOR and O_2R show several idiosyncrasies with respect to their catalytic centre. A great number of chemical, electrochemical and/or stereochemical rationalizations have been put forward to explain these particularities. In the context of this article, we would not deal with possible functional raisons d'être of one or the other feature but use them as evolutionary markers just as we have done above for the structural outlines of the enzymes in general. The two following features distinguish the catalytic centres of O_2R from those of NOR as deduced from structure and biochemical characterizations (figure 3, centre):

— NORs appear to contain Fe in the non-haem metal site, whereas O_2Rs use Cu, and
— O_2Rs feature an additional conserved residue, a tyrosine (Y), in the second coordination sphere of the Cu atom. In all characterized enzymes, this tyrosine is covalently attached to one of the histidines ligating the copper ion [53,54].

5.4.1. The non-haem metal site

In line with biochemical evidences for NORs in general, the three-dimensional structure of cNOR shows that an iron atom occupies the non-haem metal site of its BNC. The qNOR structure resolution admittedly detects a Zn atom in this position but all available evidence indicates that this is an artefact due to the crystallization procedure [49]. As was predicted by Saraste and co-workers [42], long before the three-dimensional structure, on the basis of coordination chemistry differences between Cu and Fe, the NOR non-haem metal site features in addition to the three canonical histidines a further ligating amino acid residue, a glutamate (E211 in cNOR and likely E512 in qNOR). This fourth coordinating bond afforded by the glutamate residue stabilizes Fe over Cu in the non-haem metal site. Stimulated by the structural conservation of this residue between qNOR and cNOR, we inspected the multiple sequence alignment of our updated inventory of NOR sequences. The E-residue in the sixth TM helix is conserved in all NOR-related sequences we have retrieved. We interpret this conservation as strongly indicating that iron is the non-haem metal in all representatives of the cNOR and qNOR clades. Does this mean that there is a functional constraint requiring Fe for NO- and Cu for O_2-reduction? An interesting clue to this question comes from an enzyme functioning as a NOR in *Bacillus* (*B.*) *azotoformans* [55]. This enzyme contains a cupredoxin as extrinsic subunit and when we insert the available partial sequence of its catalytic subunit into our phylogenetic reconstruction, the enzyme turns out to be part of the SoxB

(B) clade. However, it clearly carries Fe in the non-haem site of its BNC [56] and, as mentioned, physiologically functions as a NOR. The *B. azotoformans* enzyme therefore is an ex-O_2R that has con(/re?)verted to the NOR-state. At first glance, this might be taken as quite compelling evidence that NO-reduction requires iron in the non-haem metal site. However, we would suggest caution in concluding on an exclusively catalytic reason for the preference of NOR for Fe. As discussed in the first section of this article, NO reduction in general is part of a (more or less extended) denitrifying chain. Such a chain is physiologically operating when O_2 is scarce or absent [28]. Under such conditions, corresponding to an on average substantially lower ambient redox potential, copper generally is less bioavailable due to its low solubility in the reduced state. One may argue that the *B. azotoformans* enzyme in its purified form does contain copper in the Cu_A-site of its SUII [55] and that therefore scarcity of copper cannot be the problem. However, this enzyme intriguingly functions much better as a qNOR than as a cNOR [57] which may indicate that it is adapted to doing without a functional Cu_A-site. We therefore still hold that the preference of NORs for Fe may well be driven at least partly by copper-availability constraints rather than only by catalytic advantages of iron in the site. The fact that many Cu_B-containing O_2Rs exhibit substantial NOR activity [58–61] further comforts us in this view.

5.4.2. The redox active tyrosine

A tyrosine residue is seen in all O_2R structures solved so far to be cross-linked to one of the histidine ligands of Cu_B in the non-haem metal site (figure 3). The presence of this tyrosine is crucial for proper functioning of the enzyme as an O_2R [53,54]. In contrast to the glutamate residue ligating Fe in the non-haem metal site of NOR which is fully sequence conserved in all NOR clades, the tyrosine, albeit spatially in roughly equivalent locations, is found on two distinct positions in the amino acid sequence of O_2Rs. This sequence dichotomy exactly corresponds to the SoxM/B (A/B) versus cbb_3 (C) cleavage [11]. The scenario that a SoxM/B (A/B) O_2R has its tyrosine in one spot and cbb_3 (C) in the other is only tainted by the existence of several SoxB (B)-type enzymes which have neither one nor the other Y-residue [11]. To the best of our knowledge, none of these 'aberrant' SoxB (B) enzymes has so far been studied on the bench. We have in the past speculated [11] that these 'Y-less' clades might have a substituting tyrosine residue elsewhere in the sequence but again spatially close to Cu_B. The adding of new sequences to the multiple alignments does not support this model since the proposed tyrosines are not fully conserved anymore. However, the above-mentioned emerging split of SoxB (B) into two distinct clades [14,15] adds a new twist to the tyrosine story. The group of enzymes now detached from the bulk of SoxB (B) are all devoid of the canonical Sox-tyrosine and do not contain the cbb_3 (C) tyrosine either—except for the lowest branching representatives which look just like an ordinary SoxB (B) enzyme. The emerging picture therefore now argues for an additional SoxB (B) clade which ancestrally was a typical SoxB (B)-type O_2R but whereof most later-branching representatives have lost the Y-residue, and possibly their function as O_2-reductases. As they do not feature the NOR-specific glutamate residue, they likely do not represent NORs, either. Biochemical characterizations of enzymes from this new 'SoxB (B)-prime' group are therefore badly needed.

6. A structure-guided view at phylogeny

As mentioned previously, the evolutionary interpretation of the phylogenetic tree reconstructed from multiple sequence alignments of the catalytic subunits of NORs and O_2Rs is highly controversial. In the following, we will confront the phylogenetic tree with the above detailed structural particularities of individual enzyme subgroups to possibly extract information pertinent to these controversies. However, as the ensemble of NOR sequences has substantially grown recently, we will first discuss the presently emerging phylogeny of the NOR enzymes.

6.1. The expanding universe of NORs

Early genome surveys suggested a relatively small number of NORs and in particular a suspiciously restricted species distribution of cNORs which seemed limited to crown-group (i.e. α-, β- and γ-) proteobacteria [11]. In most recent trees [14,15], a substantial increase of branches in the NOR groups can be seen but it is still claimed that cNORs 'are mainly confined to the proteobacteria lineages' [15]. Based on our own inventory of NOR-genes in the genome databases, this affirmation does not seem tenable any more. There are in fact cNORs in Clostridia, the FBC phylum, Aquificales and the Thermus/Deinococcus phylum (figure 7*b*). As if this were not enough to do away with the previously perceived narrow prevalence of cNORs, an archaeal cNOR present in the Euryarchaeon *Ferroglobus placidus* recently entered the scene.

This strong increase in representatives of qNORs and cNORs, however, renders phylogenetic reconstruction a bit tricky at the present time. We have in fact observed that the topology of the cNOR subtree changes significantly with each new sequence added to the multiple alignment, and we therefore refrain from showing the composite phylogeny of the entire superfamily since the branching order within the NOR clade as well as its rooting by O_2Rs in our opinion is likely to change in the future. To illustrate the general groupings, we nevertheless show an unrooted tree of the NOR subfamily (figure 7*b*) cautioning the reader to keep in mind that the ordering of the major clades is probably not the last word, whereas the cladistic grouping by itself appears to be relatively robust.

The phylogenetic clustering into cNORs and qNORs seen in figure 7*b* is confirmed by the observation that all members of the cNOR clade contain a gene coding for a *c*-type cytochrome upstream of their catalytic subunit's gene, whereas all cladistically defined qNORs feature the N-terminal hydrophilic extension which is devoid of a CXXCH motif and contain the conserved glutamate residue involved in quinone binding as seen in the structure of the qNOR from *G. stearothermophilus* [48,49].

In contrast to the cNOR subtree, the topology of the qNOR tree strongly resembles that seen previously [11]. In particular, the clear cut split into an archaeal and a bacterial clade persists despite the addition of numerous new sequences. As mentioned before, the occurrence of haloarchaeal amidst the bacterial branches does not invalidate the Archaea/Bacteria dichotomy given the massive import of genes from Bacteria into haloarchaea [35,36].

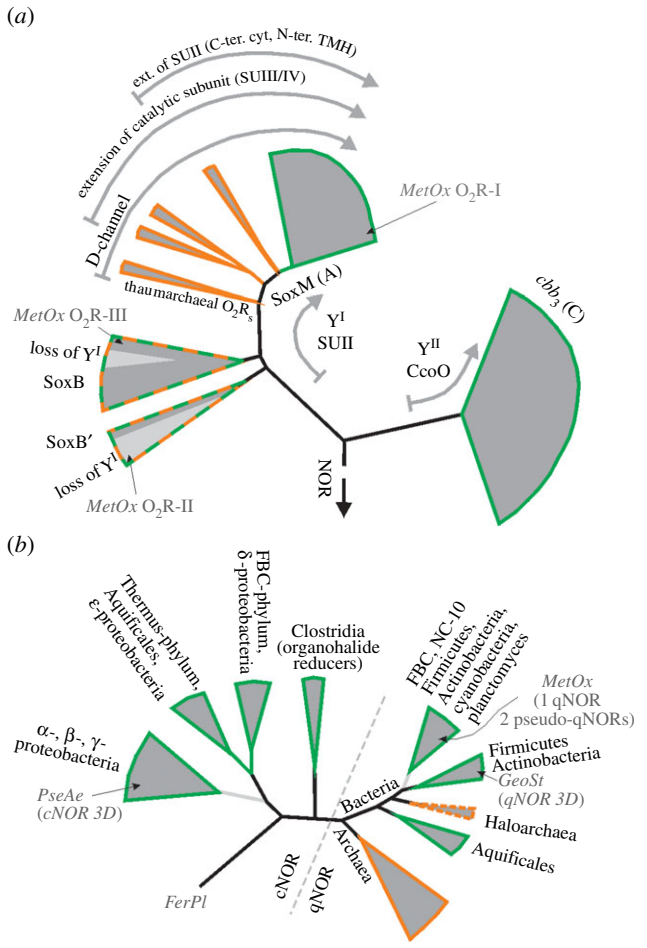

Figure 7. (*a*) Schematic phylogenetic tree of O_2Rs comprising the most recent survey of representatives and featuring structural particularities discussed in the text superimposed onto the tree. The underlying multiple sequence alignment and the tree-building procedures are detailed in the electronic supplementary material section. The phylogenetic positions of the peculiar O_2Rs from the putatively oxygenic methanotroph *M. oxyfera* (abbreviated as *MetOx*), mentioned in the text, are indicated. (*b*) Schematic phylogenetic tree of NORs based on our survey of available genomes. The phylogenetic positions of the two NORs for which three-dimensional structures are available and which are extensively discussed in this review are indicated as well as those of the putative qNORs present in the *M. oxyfera* genome. The lighter branches in (*b*) denote groups that lack the central proton conducting residues of a putative K-channel as discussed in §5.3. Whereas all members of the proteobacteria cNOR clade appear to lack the K-channel, its absence is not shared by all members of the respective qNOR clade. Internal bootstrap values in this clade, however, are too low to permit a reliable phylogenetic detailing of the K-channel deficiency. Bacterial and archaeal groups are marked by green and orange outlines, respectively, both in (*a*) and in (*b*). The green-orange dashed outlines of the SoxB and SoxB′ clades in (*a*) indicate that both archaeal and bacterial representatives make up these clades. The outline of the haloarchaeal clade in (*b*) is dashed to emphasize that haloarchaea contain an extraordinarily high number of bacterial genes as discussed in the text. *PseAe*, *PerPl* and *GeoSt* stand for *P. aeruginosa*, *F. placidus* and *G. stearothermophilus*, respectively.

The absence of the K-channel in the α-proteobacterial NORs and possibly in the FBC- and NC-10-containing phylum of qNOR as deduced from sequence comparisons is indicated in figure 7*b* by lighter branches. The cladistic positions of the functionally intriguing NOR enzymes from *M. oxyfera* as well as the NORs from *G. stearothermophilus* and *P. aeruginosa*, for which three-dimensional structures are available, are marked by arrows.

In early phylogenies of the NOR/O_2R superfamily, the enzyme from the Clostridium *Desulfitobacterium* (*D.*) *hafniense* was already found somewhat intermediate between qNORs and cNORs (e.g. [11]). A number of clostridial genomes are now sequenced demonstrating that the outlier position of the *D. hafniense* cNOR is not a feature of the clade of the Clostridia since several clostridial NORs cluster nicely within their parent phylum of the Firmicutes. Strikingly, however, the outlier clade appears to be correlated to a specific type of metabolism, i.e. organohalide respiration. Organohalide respiration is a poorly understood bioenergetic mechanism which is of utmost biotechnological importance. Organohalides are among the most widespread and nastiest industrial pollutants which modern society has to deal with [62–65]. Most organohalides are considered xenobiotics and organohalide respiration may therefore represent a recent evolutionary innovation. The isolated phylogenetic position of the respective cNORs may thus artefactually arise from rapid evolutionary adaptations. The functional role of cNOR in this group of very specialized microorganisms appears to be an exciting topic for future research.

To our minds, the persisting Archaea/Bacteria dichotomy of qNORs and the ever-increasing coverage of major phyla by members of the cNOR group strengthen the case for NORs having been present prior to the Archaea/Bacteria divergence as proposed before [11]. This case is obviously stronger for qNOR than for cNOR.

It was previously argued that the scant species prevalence of NORs in general and cNORs in particular disqualifies them as being an evolutionarily ancient enzyme [13], a reasoning often quoted as 'the phylogenomic criterion'. We have in the past argued that the underlying logic of this reasoning escapes our understanding [66,67]. The fact that the environment of present-day planet Earth is certainly substantially different from its pristine counterpart to our minds predicts that major biological players on the early planet would have a hard time in most extant habitats and therefore will (i) likely be rare and (ii) only show up when 'extreme' environments are sampled, if they have not gone completely extinct. As medical and biotechnological rather than evolutionary aspects predominantly guide the choice of genome sequencing projects, the increase in availability of data on organisms thriving in exotic habitats can be expected to be slow. Nevertheless, as shown in figure 7*b*, these data do come in and it seems to us that the very data foundation of the phylogenomic argument discarding NOR as ancestral enzyme is in the process of disappearing.

6.2. A smooth transition from SoxB (B)- to SoxM (A)-type O_2Rs

In figure 7*a*, we have summarized the main structural and functional idiosyncrasies of the various subgroups of the O_2R families by superimposing them on a schematized phylogenetic tree. This tree essentially corresponds to that published recently by Sousa *et al*. [15]. One recently added clade, however, deserves special attention. As has been noted in the analysis of the genome sequence from the Thaumarchaeon *Nitrosopumilus* (*N.*) *maritimus* [68], an O_2R present in this organism looks like a chimaera of a SoxB (B)- and a SoxM (A)-type enzyme. It contains the D-channel residues but is devoid of a SUIII/IV-related polypeptide chain and its SUII is still of the minimal cupredoxin-type just as in SoxB (B)-type sequences. A genomic search yields a number of

further sequences sharing the features of the *N. maritimus* enzyme all of which are from thaumarchaeal species. Adding these sequences to the tree reconstruction procedure yields a new clade which branches lower than the canonical SoxM (A) group towards SoxB (B) (and the possibly distinct SoxB′, see above) clade.

Since the first large-scale genome surveys [8], it has been noted that the SoxM (A) clade actually falls into two distinct subgroups, i.e. a compact, monophyletic cluster containing mainly bacterial sequences and a polyphyletic part featuring several individual clades towards the lower part of the SoxM (A) tree. Only the *ad hoc* positioning of the SoxM (A) root between archaeal and bacterial branches yields a monophyletic form of the archaeal branches. All phylogenies relying on reconstruction based on representatives from the entire superfamily ever since the first comprehensive survey in 2001 [8] observe a succession of archaeal clades branching individually prior to the node representing the origin of the bacterial radiation. The lowest two of these clades are represented in figure 7*a*. Stimulated by the intriguing 'intermediary' characteristics of the thaumarchaeal sequences, we re-examined the corresponding properties of the two clades branching in between the thaumarchaeal branches and those belonging to the typical SoxM (A)-type enzymes. It turns out that the first of these clades (i.e. that which is closer to the thaumarchaeal branches) features enzymes that possess a SUIII/IV-related membrane-integral protein domain but still stick to the minimal cupredoxin motif for their SUIIs. In the next clade, SUII does contain both the second N-terminal TM helix and the C-terminal extension, that is, this clade finally has all canonical features of SoxM (A)-type O_2Rs.

The pattern emerging from figure 7*a* therefore casts serious doubts on the validity of a sharp limit between SoxB (B)- and SoxM (A)-type enzymes. To us, this figure suggests the inception of the Sox-type O_2Rs when the tyrosine (Y^I) and the cupredoxin-type SUII (and possibly but not necessarily Cu in the non-haem metal site) appeared in the enzyme. This enzyme architecture was then in the course of evolution amended by the sequential addition of first the D-channel, then the extension of the catalytic subunit by SUIII/IV-related TM helices and finally the addition of the second N-terminal TM helix and of a solvent-exposed C-terminal domain in SUII. Although it makes perfect sense to distinguish between SoxM (A) and SoxB (B) with respect to the functional parameter of substrate affinity (SoxM and SoxB enzymes are generally considered to exhibit low and high affinity for their substrate O_2, respectively), a classification scheme putting SoxM (A) and SoxB (B) both on the same level as cbb_3 (C)-type enzymes (see §3) is not supported by figure 7*a*. Incidentally, at present there is no empirical evidence indicating where the 'affinity-border' between high- and low-affinity O_2Rs needs to be drawn in figure 7. We certainly would predict that the transition between high- and low-affinity might be correlated to the presence and absence, respectively, of the D-channel. However, none of the O_2Rs suggested by figure 7 to be in the twilight zone between SoxB (B) and fully equipped SoxM (A) has to our knowledge been studied on the bench so far. In this context it is necessary to point out that the often cited monophyly of the bacterial SoxM (A) clade is a myth. All bacterial sequences certainly group together. This group, however, falls into at least four different subgroups which strongly mix recognized bacterial phyla. Proteobacteria, for example, are present in all of these groups and planctomycetes, chloroflexi and deinococcales are found in two of them. The ensemble of the bacterial cluster thus looks much more like a horizontally juggled mess then an orderly vertical inheritance tree.

6.3. SoxM (A) versus Sox (B): the struggle for priority

As discussed in §4, the tree of figure 2 unfortunately resists being interpreted in a straightforward way. To overcome these difficulties, we have in the past proposed that O_2Rs have appeared at least twice or even three times independently from NOR-related ancestors giving rise to cbb_3 (C)-, SoxB (B)- and SoxM (A)-type enzymes [11]. In the light of the new information summarized in figure 7, we would further specify our scenario to 'having appeared exactly twice', yielding cbb_3 (C)-type O_2R by introducing the tyrosine we have called Y^{II} and the Sox-type enzymes through the emergence of Y^I. Both of these independent inceptions would have occurred in microaerobic environments within the Bacteria resulting in the first cbb_3 (C)-type O_2R and within the Archaea likely yielding a SoxB′-related ancestor of the Sox-family. SoxB (B) gradually transformed into SoxM (A)-type enzymes. The first step towards a genuine SoxM (A) O_2R would have been triggered by rising O_2 levels driving the emergence of the D-channel and the lowering of the affinity of the enzyme for O_2. The appearance of SoxB/B′ (B) would therefore evolutionarily precede that of SoxM (A).

By contrast, the competing scenario put forward by Brochier-Armanet and co-workers [12,13] and outlined in §4 stipulates that the low-affinity SoxM (A) O_2R came first and was present in the LUCA, that is, on the early planet, and that SoxB (B) (just as cbb_3 (C)) evolved only later from a SoxM (A)-type ancestor as a result of colonization of O_2-poor habitats. According to figure 7*a* (and inverting all arrows), on their way from SoxM (A) to SoxB (B), they would have sequentially gotten rid of SUII-extensions, SUIII/IV-related TM helices and finally the D-channel. Such an evolutionary simplification of a previously complicated enzyme may appear counterintuitive at first sight but can indeed be rationalized by the need to economize in an energy-limited world [66].

What is clear is that these two distinct scenarios entail radically different predictions on how the early Earth might have looked like at the time and in the places where life was evolving the NOR/O_2R superfamily of enzymes. We will therefore in the following try to summarize the present state of palaeogeochemical arguments with respect to the possibility of O_2 or nitrate and nitrite having served as oxidizing substrates for bioenergetic mechanisms during the times of the LUCA.

7. The history of O_2: the ups and downs of an anoxic early Earth

The standard model of palaeogeochemistry, based on the analysis of the level of mass-independent fractionation (MIF) of sulfur isotopes [69] stipulated an O_2-free early planet prior to the Great Oxidation Event (GOE) at around 2.3 billion years ago [70]. The O_2 accumulating in the atmosphere after the GOE was certainly produced by oxygenic photosynthesis which, however, does not necessarily mean that oxygenic phototrophs appeared just prior to the GOE. Palaeogeochemists have in fact long argued that the reduced redox compounds (most likely Fe^{2+}) may have buffered away

photosynthetically produced O_2 for substantial amounts of time. In support of this argument, palaeogeochemical evidences are interpreted as suggesting that transient excursions from the 'low or no' O_2 pre-GOE planet occurred as early as 2.7 billion years ago [71,72]. And these early 'whiffs of O_2' have recently been argued to date back to 3 billion years ago based on the analysis of redox-mediated mobilization of chromium isotopes [73]. Note, however, that the MIF data for the very same period still point to very low levels of O_2.

Whereas these newly emerging, increasingly precocious dates for the rise (although transient) of O_2 are certainly of paramount importance to the biologists studying the emergence of oxygenic photosynthesis, they do not impact the question whether the biological entity 'LUCA', estimated to have existed prior to 3.4 billion years ago, had the option to use O_2 as oxidizing substrate for energy conversion [1].

By contrast, the recent soul-searching of the palaeogeochemical community with respect to the significance of the term 'anoxic', triggered by the affirmation that O_2 respiration may have operated early on [74,75], certainly does as will be addressed in the following section.

7.1. How little O_2 is anoxic?

The upper limit for O_2 levels in the Early Archaean that can be derived from the MIF data corresponds to 10^{-5} of the present atmospheric level (PAL) [76]. Through the solubility coefficient of O_2, this translates to roughly 2 nM dissolved O_2 in the primordial ocean. Abiotic pathways capable of producing molecular oxygen, such as radiolysis [77,78] or UV-photolysis [79,80] have been shown to potentially produce O_2 levels likely far below but certainly not exceeding the 10^{-5} PAL threshold [76,81].

To the microbiologist, 2 nM [O_2] means anaerobic. Since Pasteur's times until today [82–84], microorganisms have been observed to switch from aerobic respiration to anaerobic types of metabolism below an O_2 concentration of about 0.5–2 μM, that is, at least 500 times higher than the upper limit of primordial O_2 levels. This switching concentration of O_2 is the 'Pasteur point'. The crucial gas sensor proteins regulating gene expression of enzymes involved in aerobic and anaerobic metabolism have been shown to swap repression/activation patterns in this very range of concentrations [85]. Below their threshold O_2-levels, the expression of genes coding for proteins involved in aerobic respiration is downregulated [86]. Does the existence of the Pasteur point thus discredit aerobic respiration in the LUCA?

7.2. Aerobic respiration below the Pasteur point?

Stimulated by the aerobic respiration-early scenarios [4,12,13], Stolper *et al*. [74] reassessed the lower limit of O_2 concentration for aerobic growth in *E. coli*. Increase of cell number was still observed below the detection limit of 3 nM [O_2]. This fact was interpreted as evidence that aerobic respiration is a viable energy source even at O_2 levels commensurate with the upper limit of the 'anoxic' primordial Earth. The reported results are somewhat difficult to reconcile with previous observations on the same species [87] and with the estimate that O_2 consumption by respiration greatly outcompetes delivery by diffusion below O_2 levels of 100 nM [85]. However, if *E. coli* can indeed grow on O_2 reduction at these low levels, it certainly does so in a rather uneasy way. Cell numbers increase linearly rather than exponentially and the apparent doubling time decreases from roughly 5 h in the early phase of the experiments to more than a day at the end, indicating that the culture approaches the stationary phase. We would argue that many anaerobic types of metabolism (such as reduction of nitrate and nitrite) would strongly outcompete aerobic respiration of this sort.

In any case, aerobic respiration at nanomolar O_2 levels does not support the evolutionary scenario which prompted these experiments. This scenario stipulates SoxM (A), i.e. the low-affinity O_2Rs, as ancestral and present in the LUCA [12,13]. Consequently, Han *et al*. [1] consider that LUCA invented the low-affinity SoxM (A) enzyme in a high O_2 environment, and the high-affinity SoxB (B)- and cbb_3 (C)-type O_2Rs would have appeared only when life started to colonize microaerobic habitats. This scenario therefore predicts a LUCA thriving at O_2 levels of higher than 1 μM. To the best of our knowledge, no geochemical evidence for such high concentrations of O_2 prior to the GOE has been reported so far.

8. The history of nitric oxide

When microorganisms find themselves confronted with oxygen tensions below the Pasteur point, they switch to anaerobic respiratory or, if they contain the enzyme repertoire, to fermentative metabolisms. A plethora of anaerobic types of respiration have been found during the last few decades ranging from the reduction of the small organic molecules DMSO and TMAO to that of toxic chemicals such as arsenate, selenate or chlorate. The most widespread of these anaerobic types of respiration, however, certainly is the denitrifying pathway. As this pathway features NOR as one of its key enzymes, palaeogeochemical data on the likelihood of the presence of its substrates nitrate and nitrite on the early Earth are again crucial to assessing the validity of evolutionary scenarios postulating the emergence of NOR in the LUCA.

What then of the nitrogen oxides? Volcanic exhalations in the Hadean were dominated by carbon dioxide that invaded an atmosphere with around twice the present volume of nitrogen [88]. Volcanoes also emitted some nitrogen oxides directly to the atmosphere [89]. Carbon dioxide partial pressures then were likely between 2 and 10 bars [75,90]. At these levels, photolysis and lightning strikes would drive the formation of NOs from CO_2 and N_2, to NO so augmenting further the atmospheric concentrations of NO_x to between 0.1 and 0.01 bar [11,89,91–95]. Atmospheric NO_x would eventually end up in the primordial ocean as the nitrogen oxyanions nitrate and nitrite [11]. That the proposed atmospheric production of NO is more than the extrapolation of laboratory experiments is intriguingly demonstrated by the remote, that is, from the Earth, detection of lightning-induced NO in the atmosphere of Venus [96]. The absence of a standing water body due to the elevated ambient temperature on Venus, however, precluded accumulation of the derived nitrogen oxyanions in a Venusian ocean. These more recent estimates on the production efficiency of NO in the atmosphere thus further support Mancinelli & McKay's 1988 [91] argument favouring the emergence of denitrification prior to aerobic respiration.

In striking contrast to the case of O_2, where palaeogeochemists were recently pushed by biological evolutionary scenarios to consider the possibility of higher concentrations than the previous estimates, for the case of the nitrogen

oxyanions it was the palaeogeochemists who first stipulated the operation of a denitrifying chain in the Hadean and the Early Archaean [91] a decade before the idea was first raised by the biologists based on phylogenetic data on the NOR/O_2R superfamily [7].

As NOR catalyses the reduction of only transiently formed NO while the environmental substrates are nitrate and/or nitrite, the odds for the presence of the remaining enzymes in the chain also need to be considered. Nar is a member of the enormous molybdo/tungsto-bisPGD superfamily [97]. A very strong likelihood (based on phylogenetic data) for this superfamily and in particular for Nar being pre-LUCA has been reported [98]. This study, however, only considered nNars. Several of the authors of this article have recently addressed the composite phylogeny of pNar and nNar (B Schoepp-Cothenet, F Baymann, A Magalon, W Nitschke 2014, unpublished data). The results of this work corroborate the likelihood of a pre-LUCA presence of the enzyme.

Two distinct enzymes can catalyse the reduction of nitrite to NO in prokaryotic denitrifying chains, i.e. the so-called Cu-Nir and the cd_1-Nir. Cu-Nir appears to be a later innovation of the prokaryotic enzyme repertoire [99] in line with the fact that it contains copper atoms as catalytic metals. The cd_1-Nir has so far been found both in Archaea and in Bacteria and their phylogeny suggests a pre-LUCA origin [99]. However, of all the enzymes in the denitrifying chain, cd_1-Nir is the least widespread and a more affirmative statement likely has to await more genome sequences and thus a better-furnished dataset. The terminal enzyme, N_2OR, however, by all standards (phylogeny and metal availability) was not present in the LUCA [99]. A hypothetical denitrifying chain in the LUCA therefore likely terminated at N_2O, a biologically inoffensive but geochemically notorious gas. N_2O has a greenhouse potential exceeding that of CO_2 by a factor of 200 [100–102]. The putative operation of an incomplete denitrification chain in the Hadean and the Early Archaean may therefore have had repercussions for the greenhouse gas balance of the early planet.

9. O_2 from within the last universal common ancestor?

As argued above, we consider the low levels of O_2 possibly produced by abiotic processes as very unlikely to have driven the emergence of aerobic respiration on the early Earth. A tantalizing new result from biology, however, potentially opens an unexpected backdoor for O_2 to enter the scene very early on. The discovery in 2010 of *Methylomirabilis* (*M.*) *oxyfera*, a prokaryote from the domain of the Bacteria, able to oxidize methane anaerobically coupled to the reduction of nitrite [103] may have turned the tables with respect to bioavailability of O_2 in anaerobic environments. This organism very likely uses the membrane-integral enzyme particulate methane monooxygenase to oxidize methane to methanol. Although exergonic, this reaction requires an activation of the very stable methane molecule prior to electron abstraction. In traditional, aerobic methanotrophs, this activation is achieved by O_2. The scarcity of molecular oxygen in the growth habitats of *M. oxyfera* obviously disqualifies environmental O_2 as activating agent. Although from chemistry the NO transiently produced further downstream in the denitrifying chain of *M. oxyfera* might be argued to fulfil the role of O_2 [67], Ettwig *et al*. [103] favour a different scenario based on oxygen isotope and gas analysis data. In their model, O_2 is produced by dismutation of two NO molecules. The enzyme proposed to be responsible for the dismutation of two NO to N_2 and O_2, is one or both of the two pseudo-qNORs that can be detected in the genome of the organism [37]. These qNORs differ from the canonical qNOR sequences by the absence of the glutamate residue involved in quinone binding and the replacement of one of the three histidines ligating the non-haem metal centre in the BNC by an asparagine. Since according to the experimentally determined reaction stoichiometries only three of the four putatively produced O_2 molecules are consumed in the activation of methane, Wu *et al*. [104] propose that the remaining molecular oxygen is used for aerobic respiration. In fact, three distinct gene clusters encoding sequences clustering with O_2Rs are present in the genome of *M. oxyfera*. Expression profiles [104] showed that only one of these, putative O_2R-II, is expressed at low levels, whereas none of the other two could be detected. The scenario put forward by Wu *et al*. [104] consequently stipulates that *M. oxyfera* respires on internally produced O_2 by means of the putative O_2R-II.

Two of the authors of the present review have indeed recently proposed that denitrifying methanotrophy related to that of *M. oxyfera* may have been operating in the LUCA and possibly even have played a crucial role in the emergence of a kind of inorganic metabolism ultimately giving birth to life [67]. The mechanism of internal O_2 production by this metabolism therefore would indeed provide scope for the inception of O_2Rs already in the LUCA while not affecting the palaeogeochemically observed O_2 levels in the environment. Wu *et al*. identify the putative O_2R-II from *M. oxyfera* as a *bo*-type quinol O_2R, based on the spectroscopic characteristics observed in membranes and on the absence of the Cu_A-binding residues on SUII. The fact that only SUI and SUII were detected in the respective gene clusters suggests that O_2R-II is in fact a SoxB (B)-type enzyme. This suspicion turned out to be fully warranted when we included O_2R-II in the phylogeny of the entire superfamily (figure 7*a*). Disconcertingly, however, a close inspection of its amino acid sequence showed that O_2R-II lacked not only the Y^I tyrosine residue (no Y^{II} was present either) but also the canonical histidine ligand of Cu_B which normally is covalently linked to Y^I. Not only is 'O_2R-II' thus probably not a low-affinity enzyme, it is doubtful whether it functions as an O_2 reductase at all. An examination of all three putative O_2R operons in *M. oxyfera* indicates that only O_2R-I is a *bona fide* O_2 reductase carrying all the crucial residues and, more precisely, a SoxM (A)-type enzyme (figure 7*a*), whereas both 'O_2R-II' and 'O_2R-III' belong to (the two possibly distinct domains of) SoxB (B) and 'O_2R-III', just as 'O_2R-II', lacks the Y^I residue calling into question their functioning as O_2 reductases altogether. In this context, it is noteworthy that only the 'true' qNOR was co-downregulated together with other enzymes of the denitrifying pathway when *M. oxyfera* was exposed to O_2 [105]. The two pseudo-qNORs were, by contrast, found to remain level or even to be upregulated under these conditions. These results are not straightforwardly explained in the scenario proposing these enzymes to produce O_2 for anaerobic methane oxidation [37].

In summary, it appears to us that both the qNOR and the O_2R-related enzymes of *M. oxyfera* are of paramount interest and deserve in-depth biochemical and functional investigations. Their sequence characteristics and clade-affiliations, however, do not support the scenario of a pre-LUCA SoxM

(A)-type high-affinity O_2R. Whether the low branching positions of the two SoxB (B)-type enzymes indicate a possible origin of Y^I-containing O_2 reductases in the framework of denitrifying methanotrophy can only be assessed after a more comprehensive understanding of *M. oxyfera*'s metabolism and its involved enzymes has been accomplished.

10. Darwin meets Boltzmann: does thermodynamics have a say in evolution?

The profound variations in bioenergetic properties, i.e. substrate affinities and proton pumping idiosyncrasies between the distinct subgroups of O_2Rs and NORs are intriguing. It is frequently argued that the very high standard redox potential of +820 mV of the O_2/H_2O couple would even at very low O_2 tensions provide sufficient driving force for O_2Rs to pump two extra protons per electron transferred to water. The distinct properties of SoxB (B)- and cbb_3 (C)-type enzymes as compared to SoxM (A) O_2Rs therefore must find their explanations elsewhere than in thermodynamics (see for example the discussion in Han *et al.* [1]). This argument seems to be even more striking for the case of NO reduction where the absence of pmf generation in cNORs of the *P. aeruginosa* kind is fully counterintuitive considering the even higher standard redox potential of the NO/N_2O couple of almost +1200 mV. So why on the Earth does cNOR disdain harvesting free energy if there is more than enough at its disposition? Advocates of the NOR-first scenario might argue that NOR being evolutionarily older, the enzyme did not yet have the time to evolve the proton pumping mechanism. The trouble with this argument is that all NORs we study today on laboratory benches had more than 3 billion years, just as other enzymes, to get all required mutations right to pump protons just as a SoxM (A)-type O_2R does. As we have seen above (§5.3), the argument would become even more obsolete if qNORs and some cNORs should use their predicted K-channel for proton pumping. If the common ancestor of NOR in fact knew how to pump protons, why did the *P. aeruginosa* enzyme lose these skills?

The thermodynamic reasoning developed for the case of the extreme acidophilic aerobic iron oxidizers such as *Acidithiobacillus* (*A.*) *ferrooxidans* [106] instils further doubts concerning the 'lack-of-thermodynamic-constraints' scenario. For *A. ferrooxidans*, a redox driving force of only 330 mV resulting from the comparatively high midpoint of the substrate, iron, was proposed to rationalize the apparent absence of the K-channel (as deduced from sequence alignments) in the organism's SoxM (A)-type O_2R and a resulting decrease in proton pumping efficiency in acidophilic iron oxidizers in general [106]. The (heterotrophic or autotrophic) reducing substrates of most bioenergetic chains admittedly feature redox potentials substantially more reducing than the Fe^{2+}/Fe^{3+} couple. However, such electron transfer chains contain further redox enzymes such as various types of substrate-oxidizing systems or Rieske/cyt*b* complexes which 'electrically', that is, with respect to redox energy, function in series. In a smoothly operating bioenergetic chain, the full redox energy available in the electrochemical disequilibrium between the reducing and the oxidizing substrate thus is distributed in individual redox drops along the chain, very much like the voltage of a battery is split over a series of resistances (or more precisely of impedances) according to Ohm's Law. This implies that

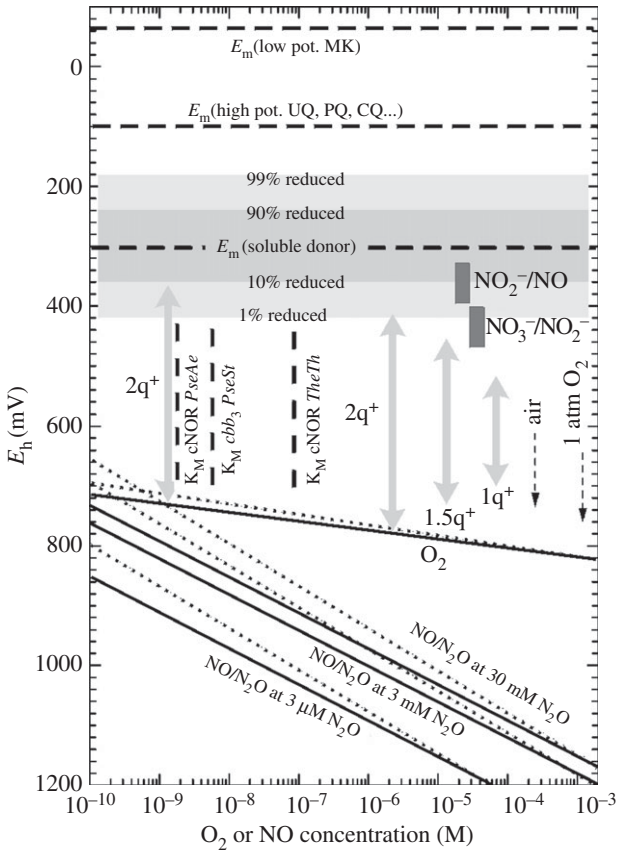

Figure 8. Schematic of the effective redox spans between electron donors to NORs/O_2Rs and the substrate couple at concentrations of O_2/NO ranging from a few millimolar down to 0.1 nM. The dotted lines represent the concentration dependence of the effective redox potentials of substrates at 70°C. Grey arrows illustrate the redox energy required to translocate 1 positive charge (corresponding to the combined movement of an electron from the P-side and a proton from the N-side to the BNC), 1.5 [1] or 2 positive charges (q) across the membrane. The redox potentials of the nitrate/nitrite and nitrite/NO couples for 10–90% reduction states are indicated by dark vertical rectangles. The grey and light grey areas correspond to the effective redox potential of a typical soluble donor with a midpoint potential of +300 mV in the 10–90% and 1–99% reduction ranges.

the available redox span for a typical cO_2R is the difference between the potential of the soluble electron donor and that of the O_2/H_2O couple. The midpoint potentials of the soluble electron donors (mono- or dihaem cytochromes, copperproteins such as amicyanin, or HiPIPs) are mostly in the range of +250 to +350 mV, and the potential of the O_2/H_2O couple is as mentioned +820 mV under standard conditions. Standard conditions mean 1 M (or 1 atm for the case of gases) substrates at 25°C and redox midpoint potential means oxidized and reduced forms of a redox compound present at equal amounts. Is the roughly 150 mV higher redox potential difference (under standard conditions) between the soluble donor and the substrate in neutrophilic aerobic respiration (+820 − 350 = 470 mV) as compared to the *A. ferrooxidans* case (330 mV, see above) enough to be sure that the thermodynamic barrier for proton pumping is out of sight?

10.1. Driving forces under conditions of an operating chain

Figure 8 attempts a summary of the ranges of values which the effective redox potentials of the relevant redox couples may

attain in operating bioenergetic chains respiring either O_2 or nitrogen oxyanions. The dependence of O_2's effective redox potential on concentration has indeed a relatively mild slope decreasing by less than 100 mV from standard conditions to nanomolar concentrations of molecular oxygen dissolved in water. Incidentally, we note that this is an even smaller decrease than that claimed by Han *et al*. [1] since standard conditions correspond to 1 atm and hence 1.25 mM of dissolved O_2 rather than to 1 M O_2. At 1 nM, the effective redox potential of O_2 is roughly +725 mV at 25°C. As temperature enters the slope coefficient ($2.3\,RT\,n^{-1}\,F^{-1}$) of this concentration dependence, the decrease is slightly more pronounced at the growth temperatures of thermophilic organisms (dotted lines for $T = 70$°C in figure 8).

The stronger dependence on concentrations at high temperatures is in fact only part of the effects of temperature on redox potentials. The standard potentials of redox compounds are defined at a given temperature (frequently 25°C) and coefficients for *T*-dependence of the standard potentials have been measured for many of these compounds [107]. Factoring in all temperature dependences, the decrease in redox potential at standard conditions and pH7 of the NO/N_2O couple with respect to the standard hydrogen electrode actually approaches 100 mV. However, most of these temperature effects apply to a more less (depending on the temperature coefficients) pronounced degree to all redox centres, that is, likely also to the soluble donors of NORs and O_2Rs. Although to our knowledge, the temperature dependences of redox potentials for these soluble donors have not been studied, they have been so for other redox proteins (e.g. [108]) and decreases in the range of those of the O_2/H_2O and NO/N_2O couples have been found. Such a general temperature dependence would therefore only downshift all redox potential values shown in figure 8 while not significantly impacting redox driving forces. For the sake of simplicity in figure 8, we have therefore restricted the illustration of *T*-dependences to that involved in changing reactant concentrations.

The soluble donors in typical bioenergetic chains are superstoichiometric with respect to the individual complexes and therefore behave like a pool of reducing equivalents the effective redox potential of which depends on its reduction state. The shaded areas in figure 8 visualize these effective redox potentials for 1, 10, 50, 90 and 99% of pool reduction state.

cO_2Rs oxidize periplasmic electron donors and the corresponding negative charge therefore moves against the pmf to the catalytic BNC of the enzyme. A proton, i.e. a positive charge, derived from the N-side of the membrane has to migrate towards the BNC, again against the pmf, to meet the electron and serve for one-fourth of O_2-reduction. The sum of the electron and proton movements is equivalent to the translocation of one positive charge across the membrane against the typically about 180 mV of pmf of an energized bioenergetic membrane. SoxM (A)-type O_2Rs in addition pump an extra proton per electron over the lipid bilayer from the N- to the P-side. The minimal total redox energy required thus amounts to 360 mV for a SoxM (A)-type enzyme. This redox span is indicated in figure 8 by the longest of the vertical, double-headed grey arrows (marked $2q^+$).

Figure 8 therefore suggests that at roughly 1 nM O_2, the thermodynamics of O_2 reduction may start to become unfavourable when the reduction state of the pool of soluble donors is inferior to 10%. Moreover, a requirement of only twice the pmf certainly represents an underestimation since for example efficient forward electron transfer from the soluble donor to the enzyme (90% of reducing equivalents proceeding to the O_2R instead of 50%) already requires at least an extra 60 mV of redox driving force.

However, how realistic are the parameters '1 nM O_2' and '10% reduced soluble donor pool' in the daily life of a bioenergetic chain? Many prokaryotes contain several O_2Rs of various types which frequently are coexpressed under specific environmental conditions. The aa_3- and ba_3-type cO_2Rs both solicit the pool of soluble donors and even the operation of a qO_2R (such as a *bo*-type enzyme) can be expected to contribute to a lowering of the reduction state of the pool of soluble donors by draining electrons from the quinone pool and thereby decreasing electron flow through the Rieske/cyt*b* complex towards the soluble donors. This situation gets even more extreme in species able to respire both O_2 and nitrogen oxyanions (such as *P. denitrificans*), where at low but finite levels of O_2 and in the presence of nitrate and/or nitrite, three more enzymes of the denitrifying chain (Nar, Nir and N_2OR) drain the pool of soluble donors (figure 1). It therefore seems to us that at least transiently low reduction states of this pool are quite plausible under conditions of growth in the natural environment.

The situation is less straightforward with respect to the concentrations of dissolved molecular oxygen. The high-affinity types of O_2R, i.e. Sox B (B) and cbb_3 (C), do feature apparent K_M values in the range of several nanomolar. However, as mentioned previously, below the Pasteur point of about 1 µM, prokaryotes prefer to switch to anaerobic types of respiration or even to fermentation. As is obvious from figure 8, the three orders of magnitude between 1 nM and 1 µM only add some 45 mV (at 25°C) to the oxidizing power of the O_2/H_2O couple and the above outlined thermodynamic limitations to pumping two protons per electron may already apply at 1 µM O_2.

However, we feel that the situation may be more complicated than that. The low apparent K_M of the high-affinity O_2Rs are indeed suspicious with respect to what is generally considered as 'microaerobic conditions', i.e. 1–5 µM O_2 [82–84]. As the operation of O_2Rs continuously consumes O_2, a respiring membrane will act as a sink for O_2, or, for those who prefer the mathematical formulation, the divergence of the scalar field $[O_2]$ (r) will be negative where there are O_2Rs. The resulting concentration gradient will initiate directional diffusion of O_2 towards the sink following the gradient vector field $\partial[O_2]/\partial x$ according to Fick's first and second laws in their vectorial versions. As the flux is linearly correlated to the concentration gradient, the replenishing of the O_2R-induced deficit slows down with decreasing ambient concentration of O_2. It has in fact been estimated [85] that below 100 nM $[O_2]$, aerobic respiration outcompetes O_2 diffusion into typical prokaryotic cells. We would argue that it is the average local concentration at the timescale of enzyme turnover that determines the effective redox potential of O_2 just as is the case in lower, O_2-depleted strata of lakes which would regain surface layer concentrations of O_2 within a few minutes through diffusion if all biological O_2 consumption were to cease. A numerical simulation of the spatial and temporal behaviour of O_2 concentrations predicted by Fick's Laws on respiring membranes is required for a realistic assessment of the effective O_2 redox potential sensed by O_2Rs under steady-state conditions of electron flow. We were unable to find such an analysis in the literature but our very preliminary attempts at such simulations indicate that local O_2 levels may

be substantially depressed with respect to ambient levels when ambient [O_2] becomes inferior to 1 µM.

The ensemble of arguments outlined above suggest to us that it may well be thermodynamic constraints rather than (or maybe in addition to) the generally proposed kinetic parameters that determine the dichotomy between low-affinity SoxM (A) cO_2Rs and high-affinity SoxB (B)- and *cbb*$_3$ (C)-type enzymes. In this context, it is intriguing that quinol-oxidizing qO_2R-type SoxM (A) enzymes are frequently found expressed under low O_2 conditions (such as for example in Bacilli). As seen from figure 8, the additional redox span gained by using a quinol as electron donor together with economizing on the movement of a negative charge against the pmf may relieve the thermodynamic limitations arising at low [O_2].

As we have already mentioned, the proton stoichiometry of high-affinity oxidases is still a matter of debate. The two competing models stipulate either an intrinsically (fixed) reduced stoichiometry of 1.5 H^+ per electron for the high-affinity enzymes [1] or a pmf-regulated stoichiometry which can attain 2H^+ per electron under appropriate conditions just as in low-affinity SoxM (A)-type enzymes which are thought to be hard-wired to a 2H^+ per electron ratio [2]. In terms of thermodynamic constraints and considering evolutionary selection pressures, a proton pumping stoichiometry working very much in the way of a friction clutch [2] would indeed make a lot of sense. When such an O_2R starts to enter a thermodynamically limited regime, sacrificing proton pumping rather than forcing the whole chain to a grinding halt is certainly advantageous. SoxM (A)-type enzymes being 'out of the woods' with respect to thermodynamic limitations, by contrast, can afford to definitively fix their proton pumping stoichiometries at the high end.

10.2. How oxidizing is nitric oxide?

The above discussed putative thermodynamic constraints certainly do not apply to the case of NO considering the exceedingly high standard redox potential of the NO/N_2O couple of almost +1200 mV. Or do they? The reduction of NO to nitrous oxide is a bimolecular reaction described by $2NO + 2H^+ + 2e^- \leftrightarrow N_2O + H_2O$, i.e. two NO are necessary to form one molecule of N_2O. According to the law of mass–action, the dependence of effective redox potentials on concentrations is therefore more complicated than the mere oxidized/reduced-ratio formula. The effective redox potential of the NO/N_2O couple is in fact described by $2.3\,RT\,n^{-1}\,F^{-1}$ times $[NO]^2/[N_2O]$. This means that the absolute concentrations of NO and N_2O are crucial and that the dependence is quadratic in [NO]. As shown in figure 8, this results in a substantially steeper drop in redox potential with decreasing [NO] than is the case for molecular oxygen and this is even more so for high temperatures (dotted lines). The dependence of the redox potential on [N_2O] is particularly interesting since many denitrifiers lack N_2OR and therefore can be expected to accumulate substantial levels of dissolved nitrous oxide. Still, the oxidizing power of NO remains comfortably above that of O_2 down to nanomolar concentrations. What then are the typical levels of NO in denitrifying organisms? As NO is toxic to cellular biochemistry, transient NO levels may be expected to remain low. Indeed, levels in the range of 0.5–5 nM have been measured in denitrifying cultures [109] which is in line with K_M values in the same range for most of the (few) studied cases [110]. As for dissolved molecular oxygen, such low concentrations entail a substantial slowing down of diffusion into NO-depleted spatial volumes and we therefore do not really know what the operating potential of the NO/N_2O couple sensed by an NOR under steady-state operations conditions might be. With respect to cNORs, rather low reduction levels (and hence high effective redox potentials) of the pool of soluble donors are not unrealistic given that at least three enzymes (figure 1) pump reducing equivalents out of this pool in typical denitrifying chains. In summary, we would argue that, rather than being exceedingly high, the effective redox potential of the NO/N_2O couple under real life conditions is likely to approach that of the O_2/H_2O couple at and below nanomolar O_2 concentrations. This still leaves enough thermodynamic room to pump protons just as the SoxB (B) and *cbb*$_3$ (C) enzymes do. Unfortunately, at the present time, the proton pumping abilities of generic NORs are uncertain. If, as the structure of the *Geobacillus* qNOR [49] and our respective sequence comparisons indicate, a K-channel may be present in specific groups of qNORs and cNORs, then these enzymes may well operate very much like their high-affinity O_2R counterparts including the use of a putative friction clutch for the pumped protons.

The cNORs of the group containing the *P. aeruginosa* and *Pseudomonas* (*P.*) *stutzeri* enzymes, by contrast, remain thermodynamic riddles. As they take both the reducing equivalent and the positively charged protons required for NO reduction from the P-side of the membrane, they are pmf-neutral and thermodynamic limitations appear highly unlikely to explain this behaviour (figure 8). The multiple sequence alignment suggests that enzymes with a disrupted K-channel occur almost exclusively in (high potential) ubiquinone-containing species, whereas those putatively carrying an intact K-channel are restricted to organisms with (low potential) menaquinone-based chemiosmotic chains. How this empirical observation can be used to rationalize differences in proton pumping capabilities remains unclear.

10.3. Thermodynamics shaped the denitrifying chain

The general layout of denitrifying bioenergetic chains as shown in figure 1 may be largely imposed by thermodynamics according to the following lines of reasoning. The redox midpoint potentials of the environmental substrates nitrate and nitrite are dangerously close to the redox midpoint potential of the pool of soluble donors as indicated in figure 8. If, as suggested by molecular phylogeny (Schoepp-Cothenet, unpublished data), pNar (fed with electrons by the pool of soluble donors) evolutionarily preceded nNar (which is reduced by quinols), the electron donor in the ancestral nitrate reducing system would have been the pool of soluble donors (figure 1). In such a system, nitrate reduction would be quickly at risk of stalling through build-up of nitrite and concomitant increase of the effective potential of the substrate couple (figure 8) unless this nitrite was further reduced. This reaction, carried out by one of the two distinct nitrite reductases (Nir), however, runs into thermodynamic limitations even more readily due to the still lower redox midpoint potential of the NO_2^-/NO couple (+360 mV) if build-up of NO would be allowed. Thus, in addition to its toxic effect, rapid removal of NO from the reactant mixture is thermodynamically mandatory for smooth steady-state respiration of nitrate and nitrite. The enzyme NOR, however, is not easily thermodynamically compromised

due to the much higher (but still lower than frequently claimed) redox potential of the NO/N_2O couple and might even have enough thermodynamic leisure to pump an extra proton. Once the initial nitrogen oxyanion substrates are reduced to the state of the biologically relatively inoffensive gas N_2O, evolution can in principle lay back and just let go of N_2O and this is what many denitrifiers do (with potentially catastrophic effects to global climate—now and then). As illustrated by the concentration dependence at different concentrations of N_2O in figure 8, additional redox pull can nevertheless be gained by further reducing N_2O to N_2. Reduction of nitrate and/or nitrite all the way through to dinitrogen therefore not only makes thermodynamic sense but also complies with the imperatives of the Gaia principle [111,112] by restoring homeostasis of the Life/Earth system. Astonishingly, the enzyme performing N_2O reduction to N_2 may have appeared comparatively late in evolution. Both its molecular phylogeny [99] and the fact that it is a copper enzyme point to an emergence of N_2OR only after the Archaea/Bacteria divergence and likely even only after the rise of O_2 levels during the GOE. If, as we surmise, denitrification was operating prior to the GOE, an absence of N_2OR might have far-reaching consequences for the greenhouse balance of the primordial planet.

11. A dire fate for the existing evolutionary scenarios?

As pointed out on several occasions in this article, all major scenarios proposed for the evolutionary history of NORs/O_2Rs envisage the presence of either an O_2R or a NOR in the LUCA, i.e. prior to the Archaea/Bacteria split. However, based on the presence of distinct haem biosynthesis pathways in Archaea and Bacteria, it was recently proposed that the LUCA may have been devoid of haem proteins [113,114]. If this picture of the LUCA were to be corroborated by future research, all presently proposed scenarios for the origin and evolution of the NOR/O_2R superfamily face serious problems. Under these circumstances, an entirely novel rationalization for the peculiar characteristics of the phylogenetic tree of these enzymes must be looked for. As argued recently [115], we consider that emerging results may indicate that the archaeal haem biosynthesis pathway may be ancestral and pre-LUCA which would allow for a haem-carrying LUCA. Alternatively, abiotically synthesized porphyrins [116,117] may also provide an emergency exit to this problem.

Whatever the eventual outcome of this recent controversy, the quickly shifting luck of evolutionary scenarios based solely on molecular phylogeny reinforce our conviction that all kinds of available 'hard' biological facts, that is, function, structure, ecology, geochemistry and thermodynamics, need to be taken into account when trying to infer deep evolutionary histories of bioenergetic enzymes. This is what we have tried to compile in this article.

12. The evolutionary history of NOR/O_2R as seen by us

All these caveats mentioned, all phylogenetic weaknesses acknowledged, we will in the following briefly outline the evolutionary scenario which to us seems to emerge from the presented results and arguments.

Our scenario envisages the origin of NOR in the LUCA and its initial operation in a denitrifying chain [11] which might have used methane as electron donor [67] and nitrate and/or nitrite produced from atmospheric chemistry as electron acceptors [91,118]. qNOR seems likely to have existed in the LUCA, whereas the data for cNOR, while having considerably expanded since previous surveys, are still relatively inconclusive. The ancestral NOR in the LUCA may have pumped protons very much like extant cbb_3 (C) and SoxB (B) O_2Rs do. High-affinity O_2Rs would then have originated twice independently from NOR-related ancestors; once in the Bacteria (giving rise to cbb_3 (C)-type enzymes) and once in Archaea (yielding the ancestor of all Sox-type enzymes) after sufficient levels of O_2 had accumulated in the environment. The inception of the two different lineages of high-affinity O_2Rs was accompanied by the recruitment of the catalytic tyrosine Y^I together with the cupredoxin-type SUII in Sox(B) and of Y^{II} and possibly a class I cytochrome as extrinsic subunit in cbb_3 (C). Alternatively, but less likely in our opinion (due to the observed low structural similarity, see figure 4), this class I cytochrome may also be an evolutionary offspring of the extrinsic subunit of NORs.

So far, all these enzymes, NOR and high-affinity O_2Rs would have contented themselves with a K-channel to transport both substrate and pumped protons. Taking sides for the proton pumping model proposed by Rauhamäki & Wikström [2], we can imagine that these NORs and O_2Rs occasionally ran into thermodynamic barriers and therefore needed a friction-clutch-type mechanism for pumping protons. Further increasing levels of O_2 after the GOE then did away with the thermodynamic limitations allowing O_2Rs to replace the friction clutch by a cogwheel and thereby fixing their proton pumping stoichiometry to $2H^+$ per electron through the addition of the D-channel and modifying details of the catalytic reaction. In a stepwise manner, on the way to the mitochondrial type SoxM (A) enzyme, additional subunits were added both in the membrane and at the P-side-exposed face of the enzyme.

Of course, it may all have been very different.

Still, we are confident that bench-analyses (with respect to O_2 affinities, proton pumping properties and three-dimensional structures) of phylogenetically distant NORs and O_2Rs as well as progress in palaeogeochemistry will eventually yield an unambiguous answer to the question of the evolutionary history of aerobic and anaerobic respiration. Such an unambiguous answer will provide crucial boundary conditions for research aiming to retrodict the evolutionary history of life even further back than the LUCA, i.e. towards life's very origin.

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