Bacterial oxygen production in the dark

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Nitric oxide (NO) and nitrous oxide (N₂O) are among nature’s most powerful electron acceptors. In recent years it became clear that microorganisms can take advantage of the oxidizing power of these compounds to degrade aliphatic and aromatic hydrocarbons. For two unrelated bacterial species, the “NC10” phylum bacterium “Candidatus Methylomirabilis oxyfera” and the γ-proteobacterial strain HN1 it has been suggested that under anoxic conditions with nitrate and/or nitrite, monooxygenases are used for methane and hexadecane oxidation, respectively. No degradation was observed with nitrous oxide only. Similarly, “aerobic” pathways for hydrocarbon degradation are employed by per/chlorate-reducing bacteria, which are known to produce oxygen from chlorite (ClO₂⁻).

In this article, we review the current knowledge about intra-aerobic pathways, their potential presence in other organisms, and identify candidate enzymes related to quinol-dependent NO reductases (qNORs) that might be involved in the formation of oxygen.

Keywords: oxygen production, nitric oxide, nitric oxide reductase, chlorate reduction, chlorite dismutase, Cld, “Candidatus Methylomirabilis oxyfera”, strain HN1

INTRODUCTION

In dim anoxic waters of stratified lakes where oxygen-respiring organisms normally cannot survive, a tiny aerobic eukaryote nevertheless makes a living. This heterotrophic ciliate, Histiobalantium natans, can survive without external oxygen because it sequesters chloroplasts from ingested euglenoid flagellates (Phacus suecicus). The chloroplasts, kept active in the ciliate and surrounded in the mitochondria, photosynthesize and produce oxygen that allows the host to thrive in deep waters of stratified lakes, where it avoids metazoan predation and competition with other aerobic ciliates (Estaban et al., 2009). This is just one example of nature’s many twists that allow organisms to take a specific niche: If an essential compound is not available, make it yourself by inventing a variation on a general theme.

For a long time, photosynthesis was the only biological process known to produce oxygen. Cyanobacteria, green plants, and algae use light energy to split water (E° = +0.82 V) via photosystem II. The electrons obtained serve NADPH and ATP generation for carbon dioxide fixation; oxygen is a mere by-product of this metabolism. This pathway evolved at least 2.7 billion years ago (Canfield, 2005), and, after the vast pools of reduced compounds on early earth were exhausted, oxygen started to accumulate in the atmosphere around 2.45 billion years ago (Holland, 2006). As a consequence, organisms evolved numerous mechanisms to cope with and/or exploit its strong oxidative properties. To prevent oxidative damage by reactive oxygen species (ROS) like superoxide (O₂⁻), hydrogen peroxide (H₂O₂), or the most damaging of all, the hydroxyl radical (OH·), detoxification systems, which often result in the regeneration of oxygen (e.g., by catalase or superoxide dismutase) evolved. These reactions have been studied and reviewed in detail elsewhere (Apel and Hirt, 2004; Murphy, 2009) and are beyond the scope of this article.

On the other hand, a large number of extant organisms are completely dependent on oxygen as the terminal electron acceptor for respiration. In addition, oxygen is the substrate in an enormous variety of monooxygenase and dioxygenase reactions that under anoxic conditions with nitrate and/or nitrite, monooxygenases are used for methane and hexadecane oxidation, respectively. The underlying mechanism is still elusive. In this perspective, we review the current knowledge about intra-aerobic pathways, their potential presence in other organisms, and identify candidate enzymes related to quinol-dependent NO reductases (qNORs) that might be involved in the formation of oxygen.
OXYGEN PRODUCTION IN CHLORATE-REDUCING BACTERIA

The first group of oxygenic chemotrophs identified were perchlo- rate and chlorate respiring bacteria (Ridken et al., 1996; van Ginkel et al., 1996). These organisms reduce perchlorate (ClO₄⁻) and/or chlorate (ClO₃⁻) to chloride (Cl⁻). Rather than being further reduced to hypochlorite (ClO₂⁻), chlorite is converted into chloro- ride (ClO⁻) and O₂. Perchlorate occurs naturally, but rarely in the environment, with significant concentrations only found in the Chilean salpeter deposits (Beckurts, 1886; Ericksen, 1983). In contrast, chlorate and chlorite are continuously generated in trace amounts in the ecosystem, much broader than could be expected from the known natural sources and the short timeframe of anthropogenic contamination (Coates et al., 1999). It now becomes clear that perchlorate is continuously generated in trace amounts in the atmosphere. Accumulation to measurable amounts, however, only occurs where deposition is high, but leaching and microbial reduction is low in an extremely arid climate (Rajagopalan et al., 2006; Xu et al., 2003). An initial surprise was the widespread occurrence of (per)chlorate reduction among microorganisms and in different ecosystems, much broader than could be expected from the known natural sources and the short timeframe of anthropogenic contamination (Coates et al., 2001). However, signature genes of anaerobic hydrocarbon activation (Heider, 2007), like the glycol-radical enzyme benzylic-succinate synthase cluster, are missing. In contrast, the genome of strain RCB only encodes genes for the aerobic activation of aromatic compounds, including several monooxygenases and dioxygenases (Salinero et al., 2009). Physiological experiments under nitrate-reducing conditions strongly suggest the involvement of a hydroxyl radical-mediated activation leading to phenol as primary intermediate (Chakraborty and Coates, 2005). It is quite unlikely that the very substrate-specific Cld can catalyze O₂ production from nitrogen oxide intermediates. This possibility has been negatively tested for NO with the recombinant Cld of Nitrosopumilus maritimus (Maxiner et al., 2008), which was also found to be inhibited by NO (179 ± 13 μM) (F. Maxiner and K. Ertwig, unpublished results). The open question is: Can the oxidative power for the attack on benzene come from oxygen, also under denitrifying conditions (Weelink et al., 2010)?

OXYGEN PRODUCTION FROM NITROGEN OXIDES?

The idea that oxygen may be an intermediate of denitrifying, anaerobic bacteria emerged when the genome of the anaerobic methane-oxidizing bacterium "Candidatus Methylosporium oxyfera" was assembled from enrichment culture metagenomes. These freshwater enrichment cultures (Raghoebar et al., 2006; Ertwig et al., 2009) couples complete methane oxidation with CO₂ as the end product to the reduction of nitrate (NO₃⁻) to nitrogen (N₂) according to Eq. 2.

$$3 \text{CH}_4 + 8 \text{NO}_3^- + 8 \text{H}^+ \rightarrow 3 \text{CO}_2 + 4 \text{N}_2 + 10 \text{H}_2\text{O} (\Delta G^\circ = -928 \text{kJ mol}^{-1} \text{CH}_4)$$

Methane has the second highest activation energy (after benzene) of all organic compounds. One of the prime questions was how it could be enzymatically activated under anaerobic
conditions. Generally, two enzymatic activation mechanisms were already known: Aerobic methane-oxidizing bacteria (MOB) employ a monooxygenase reaction yielding methanol as the first intermediate (Hakemian and Rosenzweig, 2007; Itono and Murrell, 2008). Anaerobic methanotrophic archaea (ANME), that couple methane oxidation to sulfate reduction (most likely by a monooxygenase reaction yielding methanol as the first intermediate (Hakemian and Rosenzweig, 2007; Trotsenko and Troitskaya, 2008). Anaerobic methanotrophic archaea (ANME), that couple methane oxidation to sulfate reduction (most likely performed in association with sulfate-reducing bacteria) reverse the last step of methanogenesis catalyzed by methyl-coenzyme M reductase (Knittel and Boetius, 2009; Scheller et al., 2010).

The last step, nitrous oxide reduction, is not always present, leaving the potent greenhouse gas N2O as the end product (Stein, 2011). Thus, a second surprising finding was the apparent lack of an identifiable nitrous oxide reductase; nor, nitric oxide reductase; nos, nitrous oxide reductase; nod, nitrous oxide (N2O), and eventually dinitrogen gas (N2) by denitrification.

Whereas no homologues of the two last mentioned signature genes for anaerobic methane and hydrocarbon degradation could be identified in the genome, surprisingly the entire pathway of aerobic methane oxidation, starting with particulate methane monooxygenase (pMMO), was present, and prominently transcribed and expressed (Ettwig et al., 2010). shirt chain alkanes (ethane, propane, butane), a well-known activity of pMMO (Leadbetter and Foster, 1980; Hazen and de Bruyn, 1980). Finally, using the oxidation of propylene as a proxy for pMMO activity (Prior and Dalton, 1985), comparable rates were obtained for oxygen and nitrite as electron acceptors (Ettwig et al., 2010). Also the analysis of the denitrification pathway caused a second starting finding was the apparent lack of an identifiable nitrous oxide reductase.
reductase in the genome of *M. oxyfera*, even though it had been shown that dinitrogen gas was the end product of nitrite reduction. Despite the presence of three qNOR paralogs (see below), of which two were highly transcribed and expressed, nitrous oxide was not produced in significant amounts. Now, one of two possibilities might explain the paradoxical results: (1) activation of methane to methanol by NO, yielding N₂ as the second product of the pMMO-catalyzed reaction, (2) the disproportionation of eight NO molecules would give four oxygen molecules only three of which are consumed in the activation of methane. Residual O₂ appears to be respired by one of the terminal oxidases found in the *M. oxyfera* genome (Wu et al., 2010). Obviously, the most interesting question now is the identity of the enzyme that catalyzes oxygen and nitrogen formation from NO.

The intermediary role for oxygen in the activation of recal-citrant compounds during denitrification may not be limited to *M. oxyfera*. The facultatively denitrifying γ-proteobacterium strain HdN1 grows on a wide variety of substrates, including C6- to C20-alkanes (Ehrenreich et al., 2000; Zedelius et al., 2010). Growth on hexadecane was observed with oxygen, nitrate, or nitrite as electron acceptors, but not with N₂O. In contrast, N₂O did serve as a substrate for growth on the corresponding easier-to-degrade C16-alcohol and fatty acid, which do not require oxidative activation (Zedelius et al., 2010). Like *M. oxyfera*, the HdN1 genome did not contain recognizable genes for the glycyl-radical-catalyzed activation of alkanes, such as alkylsuccinate synthase. Instead, two or possibly three monooxygenases were encoded in the genome. These findings suggest that the activation of the alkane substrate in *M. oxyfera* and HdN1 take place by a similar mechanism involving oxygen, formed from nitrate or nitrite (Figure 1B).

**DIVERGENT NITRIC OXIDE REDUCTASES IN *M. OXYFERA* AND OTHER DENITRIFYING MICROORGANISMS**

Like oxygen, NO is a strongly oxidizing compound and most microorganisms that have to deal with it as an intermediate or in their environment have developed a repertory of enzymes that convert it into the harmless N₂O as fast as possible (Richardson, 2000; de Vries and Schröder, 2002; W atmough et al., 2009). Collectively, the bacterial nitric oxide reductases (NORs) belong to the superfamily of heme-copper oxidases (HCOs; Figure 2). Members of the family share the presence of a heme b (or a) for electron transfer, and a second heme (b₃, a₁, or a₂), that together with an iron (Fe₃ or NO) or a copper ion (Cu₃ or oxidases) constitute the catalytic center. Both Fe₃ and Cu₃ are ligated to three conserved histidines. The electron-transferring heme is coordinated by two histidines as well, while one more histidine serves as the proximal ligand to the catalytic heme. This histidine sextet is a signature for HCOs.

Nitric oxide reductases catalyze the two-electron reduction of two molecules of NO into N₂O (Eq. 4).

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2\text{NO} + 2\text{H}^+ + 2e^- \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O}
\] (4)
FIGURE 4 | Quinol-binding and catalytic sites in the qNOR structure of *Geobacillus stearothermophilus* (3AYG, Matsumoto et al., 2012; left), and amino acid sequence comparison of these sites in qNORs and putative NODs (right). Sequence accession numbers and alignment are as indicated in Figure 2. Numbering above the alignment refers to the first amino acid and corresponds to the residue numbers of *G. stearothermophilus*. Specific changes in otherwise strongly conserved residues are highlighted. (A) Quinol-binding site with a bound quinol analog, 2-heptyl hydroxyquinoline N-oxide (green molecular surface). His328 and Asp746 form hydrogen bonds with the quinol moiety and the large hydrophobic residues interact with the hydrophobic tail. (B) View of the catalytic site from the plane of the heme $b_3$. The ZnB is indicated in green and two water molecules in the coordination sphere of the ZnB are indicated as small red spheres.

The different NOR types are distinguished on the basis of the electron carrier that supplies nitric oxide reduction with reductant. Best characterized are cNORs which contain an additional cytochrome $c$ subunit for this purpose, and qNORs which use reduced quinone (quinol) as the electron donor. Of both enzymes, atomic structures have been resolved recently (Hino et al., 2010; Matsumoto et al., 2012).

As mentioned above, the *M. oxyfera* genome contained three qNOR paralogs (EC 1.7.5.2, DAMO_1889, DAMO_2434, and DAMO_2437), in stark contrast to the lack of appreciable N$_2$O production during nitrite-dependent methane oxidation (Raghoebarsing et al., 2006; Ettwig et al., 2008, 2009, 2010). DAMO_1889 was expressed in only low amounts, but the two highly similar DAMO_2434 and DAMO_2437 (84% aa identity) were among the most abundant gene products, both at the transcriptional and protein level (Ettwig et al., 2010). Detailed sequence analysis revealed that DAMO_1889 shared all important features with known qNORs, while DAMO_2434 and DAMO_2437 displayed important differences, which will be discussed in detail below. Strikingly, the unusual characteristics were consistently found in two other protein sequences available in GenBank, putative qNORs from the hexadecane-oxidizing $\gamma$-proteobacterial strain HdN1 (Zedelius et al., 2010) and from *Muricauda rustringensis*, a Flavobacterium.
that had been isolated with peptone as a carbon source from a hexadecane-oxidizing, denitrifying enrichment culture (Bruins et al., 2003). A species of the same genus, *M. aquimarina*, was recently shown to degrade hexadecane and poly cyclic aromatic hydrocarbons aerobically (Jiménez et al., 2011). Although the three organisms are only distantly related, their unusual qNOR-like genes form one separate cluster within the qNORs (Figure 2).

A similar qNOR, however, is absent from the genome of the bic membrane interior with the active site. These channels might run parallel to the membrane and connect the hydrophilic position is filled by a number of voluminous aromatic amino acids. This sequence folds at the periplasmic site as a cyt c domain like in cnOR, although a heme ε is absent. Instead, the heme ε position is filled by a number of voluminous aromatic amino acids. Two hydrophobic channels are observed in the structure that run parallel to the membrane and connect the hydrophilic membrane interior with the active site. These channels might function in substrate (NO) import and product (N2O) export. Two more features distinguish qNOR from cnOR: (1) the presence of a quinol-binding site (Figure 4A) and of a water-filled channel that likely plays a role in the supply of protons for NO reduction (Eq. 4; Matsumoto et al., 2012; Shiro et al., 2012). The channel leads from the bottom of the enzyme in the cytoplasm up to the catalytic site.

The sequence comparison of the Mn oxidase and the other unusual qNORs establish both resemblances and significant differences with respect to canonical qNORs. In DAMO_1889, all characteristics are conserved, suggesting the protein to be a genuine qNOR. Also in DAMO_2434, DAMO_2437, and their relatives the overall folding is apparently maintained with respect to the one of qNORs, as is inferred from sequence comparison and structural modeling using qNOR of *Geobacillus stearothermophilus* (PDB 3AYF and 3AYG) as the template (not shown). The arrangement of the 14 TMHs, the hydrophilic domain devoid of heme ε, all histidines except one, both putative substrate channels and a portion of the amino acids related with the H+ channel are conserved. This suggests that DAMO_2434 and its relatives, hereafter referred to as putative NOD, bind the electron-transferring heme h, the catalytic heme b2, and histidine iron (or another catalytic metal). However, in the NODs one of the coordinating histidines is consistently replaced by an asparagine (Figure 4B). Similarly, a glutamate in close vicinity to the catalytic center, which has been implied with catalysis (Thorndycroft et al., 2007; Flock et al., 2009; Hino et al., 2012; Shiro et al., 2012) is substituted by a glutamine residue. Also the amino acids lining the proposed H+ channel in qNOR have undergone several substitutions in the putative NODs. Most importantly, the unusual qNORs lack a proper quinol-binding site. Conserved residues that are assumed to constitute the quinol-binding site in qNORs are substituted for amino acids that are unlikely to provide a suitable site for quinol binding in the putative NODs (Figure 4A).

In summary, the latter apparently are unable of accepting external electrons, they have a different catalytic site and might be impeded in H+ uptake from outside the protein. Obviously, these properties compromise a role as nitric oxide reductase. The question then is what they do, presuming that they do bear an important biological function – a reasonable assumption given their high expression levels in *M. aquimarina*. It is tempting to speculate that the modified proteins can bind two NO molecules, rearrange N-O bonds with the aid of the hemes and non-heme metal (iron or otherwise), and recombine both N and O atoms such that N2 and O2 are made. In other words, the enzymes would act as an NO dismutase. At this stage, this is speculation. The proof can only come from the purification and rigorous characterization of these intriguing enzymes.
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.