Bacterial oxygen production in the dark

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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 by 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 (Esteban 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 mechanism.

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 (H2O), a process known to produce oxygen. The second, “dark” way takes advantage of oxidants with a more positive redox potential than the O2/H2O couple. Only a few redox couples are biologically relevant in this respect: hypochlorite (ClO−)/Cl− (E0 = +1.18 V), nitric oxide (NO)/N2O (E0 = +1.28 V), ClO−/Cl− (E0 = +1.08 V), nitrous oxide (N2O)/N2 (E0 = +1.36 V), nitric oxide (NO)/N2O (E0 = +1.18 V), and NO/N2 (E0 = +1.27 V). Most of these compounds are intermediates in the respiration of (per)chlorate and nitrate/nitrite, respectively. In this perspective, we review what is known and still to be learned about oxygenic pathways from chloro-oxo species and nitrogen oxides, with a focus on a hypothetical enzymatic mechanism for the hitherto elusive nitrite-driven oxygen production.
OXYGEN PRODUCTION IN CHLORATE-REDUCING BACTERIA

The first group of organic chemotrophs identified were perchlorate and chlorate respiring bacteria (Riddiken et al., 1996; van Ginkel et al., 1996). These organisms reduce perchlorate (ClO$_4^-$) and/or chlorate (ClO$_3^-$) to chlorite (ClO$_2^-$). Rather than being further reduced to hypochlorite (ClO$_2^-$), chlorite is converted into chloride (Cl$^-$) and O$_2$. Perchlorate occurs naturally, but rarely in the environment, with significant concentrations only found in the Chilean saltpeter deposits (Reckarts, 1868; Erickson, 1983). In past decades, anthropogenic contamination of chlorate and perchlorate from either the use of Chile salpeters as fertilizers, or from chemical waste (e.g., solid rocket fuel spills and explosives) has been a concern and incentive for research on microbial (per)chlorate reduction (Motzer, 2001; 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., 1999). It now has become 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., 1999). It now has become 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).

(Per)chlorate respiration in principal only requires two enzymes. At first, (per)chlorate reductase, a molybdopterin-containing respiratory reductase resembling nitrate reductase, catalyzes the reduction of perchlorate to chlorate and of chlorate to chlorite. Then, chlorite is converted in a single exergonic reaction into chloride and oxygen (Eq. 1; van Ginkel et al., 1996). This is a net disproportionation or dismutase reaction in which the chlorine atom becomes reduced and oxygen oxidized.

\[
\text{ClO}_4^- \rightarrow \text{Cl}^- + \text{O}_2 (\Delta G^\circ = -100 \text{ kJ mol}^{-1}) \quad (1)
\]

The reaction is catalyzed at a high rate and with extraordinary specificity by chlorite dismutases (Cld, EC 1.13.11.49), members of the Cld superfamily of redox enzymes (Goblirsch et al., 2011). This homohexameric or homopentameric heme $\beta$-enzyme was first purified in the nineties from the β-proteobacterium Azospira oryzae (then called strain GR-1, van Ginkel et al., 1996). The enzyme is now well characterized by the resolution of the atomic structures from several species (de Geus et al., 2009; Mebboob et al., 2009; Goblirsch et al., 2010; Kostan et al., 2010; Mlynek et al., 2011). Detailed kinetic analysis established that oxygen is not derived from water, but from chlorite itself (Lee et al., 2008; Streit and DuBois, 2008). Catalysis proceeds via an oxoferryl species and a ClO$_2^-$ anion, indicating that, after initial binding to the catalytic heme $\beta$, chlorite is first cleaved, after which both oxygen atoms recombine, yielding chloride and oxygen. Most chlorate-reducing organisms found thus far are facultative aerobes that, in absence of extracellular oxygen use the chlorite-derived oxygen for aerobic respiration, analogous to the use of chloroplasts by H. natans in the introduction.

What else to do with oxygen produced by chlorite dismutase? Surprisingly, functional chlorite dismutases have also been found in Bacteria and Archaea that cannot grow with chlorate as electron acceptor, e.g., in the nitrite-oxidizing genera Nitrospira (Maiorner et al., 2008) and Nitrobus (Mlynek et al., 2011). In the latter species, Cld is significantly smaller and present as a homodimer. The role of Cld in these organisms, however, is unclear. They may possibly confer insensitivity to chlorite, coupled with the advantage of producing oxygen for nitrite oxidation in microoxic niches. In the archaean Haloferax volcanii, chlorite dismutase is hypothesized to produce oxygen for a monoxygenase encoded in the same operon, that is involved in the biosynthesis of an antibiotic (Bah-Diniz et al., 2006). In Pseudomonas chloritidismutans, oxygen likely does not only act as an electron acceptor in respiration, but is also used for alkane activation by a monoxygenase-mediated reaction (Mebboob et al., 2009a). P. chloritidismutans is capable of respiring carbon substrates like fatty acids or alcohols with oxygen, chlorate, and nitrate, but growth on alkane is not observed with nitrate as electron acceptor. This suggests that oxygen, provided externally or from chlorate reduction, is required for the initial activation of the alkane, a hydroxylation to the corresponding alcohol (Heider, 2007).

The β-proteobacterium Dechloromonas aromatica strain RB1 can degrade benzene aerobically and anaerobically with chlorate and nitrate (Coates et al., 2001). However, signature genes of anaerobic hydrocarbon activation (Heider, 2007), like the glycyl-radical enzyme benzyl-succinate synthase cluster, are missing. In contrast, the genome of strain RB1 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 O$_2$ production from nitrogen oxide intermediates. This possibility has been negatively tested for NO with the recombinant Cld of Nitrosopumilus maritimus (Maiorner et al., 2008), which was also found to be inhibited by NO (179 μM; F. Maiorner and K. Ettwig, 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 MethyloParis styxera" was assembled from enrichment culture metagenomes. These freshwater enrichment cultures (Raghoebarsing et al, 2006; Ettwig et al., 2009) couple complete methane oxidation with CO$_2$ as the end product to the reduction of nitrate (NO$_3^-$) to dinitrogen (N$_2$) according to Eq. 2.

\[
3 \text{CH}_4 + 8 \text{NO}_3^- + 8 \text{H}^+ \rightarrow 3 \text{CO}_2 + 4 \text{N}_2 + 10 \text{H}_2\text{O} (\Delta G^\circ = -928 \text{ kJ mol}^{-1}\text{CH}_4) \quad (2)
\]

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; Trotsenko and Murrell, 2008). Anaerobic methanotrophic archaea (ANME), though energetically costly, a third possibility has also been considered: The M. oxyfera enrichment culture also oxidized other short-chain alkanes (ethane, propane, butane), a well-known activity of ANME (Leadbetter and Foster, 1980; Hazen and de Bruyn, 1989). 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 surprise. In all microbial species studied so far, denitrification proceeds in a step-wise fashion, comprising the subsequent reduction of nitrate (NO$_3^-$) to nitrite (NO$_2^-$), nitric oxide (NO), nitrous oxide (N$_2$O), and eventually dinitrogen gas (N$_2$) by dedicated reductases (Figure 1A; Zumft, 1997; Einsle and Kroneck, 2004; Tavares et al., 2006). The last step, nitrous oxide reduction, is not always present, leaving the potent greenhouse gas N$_2$O as the end product (Stein, 2011). Thus, a second startling finding was the apparent lack of an identifiable nitrous oxide homologous to benzyl- or alkyl-succinate synthase (Thauer and Shima, 2008).

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), but highly sensitive to acetylene (total inhibition at 10 μM; Ettwig et al., 2010), a known inhibitor of pMMO (Prior and Dalton, 1985). Besides methane, the M. oxyfera enrichment culture also oxidized other short-chain alkanes (ethane, propane, butane), a well-known activity of ANME (Leadbetter and Foster, 1980; Hazen and de Bruyn, 1989). 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 surprise. In all microbial species studied so far, denitrification proceeds in a step-wise fashion, comprising the subsequent reduction of nitrate (NO$_3^-$) to nitrite (NO$_2^-$), nitric oxide (NO), nitrous oxide (N$_2$O), and eventually dinitrogen gas (N$_2$) by dedicated reductases (Figure 1A; Zumft, 1997; Einsle and Kroneck, 2004; Tavares et al., 2006). The last step, nitrous oxide reduction, is not always present, leaving the potent greenhouse gas N$_2$O as the end product (Stein, 2011). Thus, a second startling finding was the apparent lack of an identifiable nitrous oxide reductase. The tree was calculated with MEGA5 (Tamura et al., 2011) and is based on alignment created with ClustalW using the default settings. The alignment was manually checked for correct alignment of conserved residues. The sequences Ooij 2434 and Ooij 2437 are consensus sequences based on the contigs obtained by iterative read mapping of the Ooij metagenome (SRR022398.2) to the genome of M. oxyfera (Dutilh et al., 2009). Accession numbers: M. oxyfera DSS33440 CBE68948, DAMO_2437 CBE68922, and DAMO_1889 CBE68923; pMMO proteobacterium HmH1 NorZ1 CBL46528 and norZ2 TP_0038969311; M. ruestringensis GUFJ98416, gonzamorenae ZP_04723508; Synechocystis sp. Nox2 BAA18796; S. aureus EGS24848; G. stearothermophilus G2PJH6; P. aeruginosa BAA32546, P. denitrificans NorB NorB NP_249215, P. denitrificans NorB NorB CBL45628, and NorZ1 CBL45628, and NorZ2 CBL45628. P. aeruginosa CoN, P. denitrificans CoN, and P. aeruginosa CoN.

![Pathways of canonical denitrification (A) and proposed N$_2$ and O$_2$ production by NO dismutation (B).](image-url)
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 NO into N₂ and O₂ (Eq. 3), analogously to the overall reaction stoichiometry (Eq. 2). Next, the bypass of N₂O as an intermediate, and the formation of labeled oxygen from ^18O-labeled nitrite could be experimentally shown (Ettwig et al., 2010). The hypothetical pathway that is consistent with all observations is shown in Figure 1B. From the experimental reaction stoichiometry (Eq. 2) it is inferred that 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 recalci-tant compounds during denitrification may not be limited to M. oxyfera. The facultatively denitrifying γ-proteobacterium strain HdB (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 HdB genome did not contain recognizable genes for the glycol 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 HdB 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, 2006; de Vries and Schröder, 2002; Wutmough et al., 2009). Collectively, the bacterial nitric oxide reductases (NORs) belong to the superfam-ily 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 (b₉, a₉, or a₉), that together with an iron (Fe₉ in NOR) or a copper ion (Cu₉ in oxidases) constitute the catalytic center. Both Fe₉ and Cu₉ 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).

\[
\text{2NO} + 2\text{H}^+ + 2e^- \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O} \quad (4)
\]
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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. 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 N2O 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 γ-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 (Bruns et al., 2001). A species of the same genus, M. aquamarina, was recently shown to degrade hexadecane and polycyclic 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 benzene-oxidizing D. aromatica strain RCB.

CHARACTERISTICS OF THE PUTATIVE NO DISMUTASES

The overall atomic structure of qNOR strongly resembles the one of cNORs and other HCOs (Hino et al., 2010; Matsumoto et al., 2012). The enzyme is composed of a membrane-spanning region with 13 trans-membrane helices (TMHs) that enclose the heme \( h \), heme \( b_2 \), and Fe\( \alpha \) moieties, which are coordinated by the conserved histidine sextet. In the qNOR structure, the latter position is occupied by a (redox-insensitive) zinc atom, which most likely is a crystallization artifact (Figure 3). A particular property of qNOR is the presence of an additional (14th) N-terminal TMH that is followed by a long hydrophilic stretch of amino acids. This sequence folds at the periplasmic site as a cyt \( c \) domain like in cNOR, although a heme \( c \) itself is absent. Instead, the heme \( c \) 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 (N\( \_2 \)) 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 M. oxyfera 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 3ATF and 3AYG) as the template (not shown). The arrangement of the 14 TMHs, the hydrophilic domain devoid of heme \( c \), 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 \( b_2 \), and non-heme 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 (Thornycroft et al., 2007; Flock et al., 2009; Hino et al., 2012) is substituted by a glutamine residue. Also the amino acids lining the proposed H\( ^+ \) channel in qNOR have undergone sev- eral 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 reductases. 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. oxyfera. 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\( \_2 \) and O atoms such that N\( \_2 \) and O\( \_2 \) 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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