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Anammox biochemistry: a tale of heme *c* proteins

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(Abstract)

Anaerobic ammonium-oxidizing (anammox) bacteria are one of the latest scientific discoveries in to the biogeochemical nitrogen cycle. These microorganisms are able to oxidize ammonium (NH_4^+) with nitrite as the oxidant, (NO_2^-), instead of oxygen and form dinitrogen (N_2) as the end product. Recent research shed a light on the biochemistry underlying anammox metabolism with two key intermediates, nitric oxide (NO) and hydrazine (N_2H_4). Substrates and intermediates are converted exploiting the catalytic and electron-transfer potentials of *c*-type heme proteins known from numerous biochemical reactions and that have gotten new functionality in anammox biochemistry. On a global scale anammox bacteria significantly contribute to the removal of fixed nitrogen from the environment and the process finds rapidly increasing interest in wastewater treatment.

Ammonium and the Environment

Ammonium (NH_4^+) is an essential and often growth-limiting nutrient for all living organisms. However, excess deposits to the environment in densely populated areas and the excessive use of fertilizers in modern agriculture results in environmental hazards such as eutrophication and global warming. It is striking that more than one third of the total nitrogen (excluding N_2 in the atmosphere) that is now present in nature originates from industrial sources, most notably the Born-Haber process [1,2]. This amount is about 3.5-fold higher than the proposed safe boundary (35 millions of tons per year) for nitrogen deposition and has been progressively growing [3].

However, ammonium can be removed by microbial activity and microorganisms that are able to convert ammonium to other fixed nitrogen compounds are known for more than one hundred years. In the presence of oxygen, ammonium can be oxidized by aerobic ammonium-oxidizing bacteria (AOB) and archaea (AOA) to nitrite (NO_2^-) [4-6], which is then converted to nitrate (NO_3^-) by nitrite-oxidizing bacteria [7]. These oxidative processes are called nitrification. In the absence of oxygen, nitrite and nitrate can be reduced to N_2 by denitrifiers or to ammonium by dissimilatory nitrite/nitrate reducing (DNRA) microorganisms [8-10]. In conventional wastewater treatment, the opportunities offered by the nitrifiers and denitrifiers are utilized to remove excess fixed nitrogen from wastewater streams.

Anammox Bacteria

Ammonium is a relatively inert compound and AOB and AOA rely on the oxidative power of oxygen to activate it into the more accessible hydroxylamine (NH_2OH) [11,12]. This presumed strict dependence on oxygen may have hampered the quest to discover microbial processes through which ammonium is converted without oxygen (anoxically). Until recently, such processes were even deemed impossible. Still, twenty years ago the important

observation was made that ammonium was apparently depleted in a pilot-scale bioreactor operated under anoxic conditions. This observation initiated the search for the responsible microorganisms [13]. By dedicated methods, these very slowly growing organisms, termed anaerobic ammonium-oxidizing (anammox) bacteria, could be enriched for further studies [14,15]. After their first identification, which enabled the design of molecular tools for their detection [16,17] more than 10 species and a vast range of subspecies have been reported for the anammox bacteria in environments where fixed nitrogen is removed in the absence of oxygen [18,19]. Anammox bacteria have even been estimated to contribute for 30-70% to the annual release of N₂ into the atmosphere [20,21]. Moreover, these microorganisms are now applied as a cost-effective and environment-friendly alternative to conventional wastewater treatment [22].

Anammox bacteria are exceptional organisms from a number of perspectives. Phylogenetically, they belong to Eubacterial Phylum of *Planctomycetes* [23,24], which are characterized by their complicated cell plan. This also holds for anammox bacteria that typically harbor a large vacuolar cell organelle, the anammoxosome, where the proteins involved in central catabolism reside (Box 1).

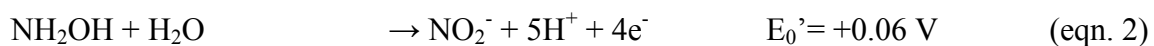
Anammox Biochemistry

Considering the presumed inert nature of ammonium, the prime question was how anammox bacteria are able to oxidize it in the absence of oxygen. Detailed analyses of substrate conversions in bioreactors [14], a chance finding regarding hydrazine (N₂H₄) [25,26], careful inspection of the first genome sequence of the anammox model organism *Kuenenia stuttgartiensis* [27], and preliminary physiological and biochemical studies [28], enabled the

formulation of a minimal set of reactions by which the two substrates of anammox bacteria, ammonium and nitrite, were converted into the end product N₂ (eqn. 1).



The conversion of the substrates would proceed in three consecutive, coupled redox reactions with two highly toxic intermediates, nitric oxide (NO) and hydrazine (Figure 1; Key Figure) [27-30]. In the first step (reaction 1 in Figure 1) of the current working hypothesis, the one-electron reduction of nitrite to NO is catalyzed by a candidate known from many denitrifying microorganisms, nitrite reductase. The second step (reaction 2) combines ammonium (or ammonia) and NO with the input of three electrons. This step would involve a biochemical novelty: an enzyme termed hydrazine synthase (HZS) and based on genome analyses a unique multiheme protein was suggested as the candidate HZS [27]. The last step (reaction 3) is the oxidation of hydrazine into N₂, which releases four electrons that drive reduction steps (1) and (2) in Figure 1. This reaction was hypothesized to be catalyzed by a variant of an already known enzyme, hydroxylamine oxidoreductase (HAO). HAO is a key enzyme used by AOB to convert hydroxylamine, the product of ammonium activation, into nitrite (eqn. 2) [31].



AOB HAO is also a multiheme protein and *in vitro* it catalyzes hydrazine oxidation as a side activity [32,33]. As such, anammox metabolism would be fully supported by heme proteins (Box 2), which would explain the typical blood red color of enrichment cultures. Although many aspects remain to be resolved, ongoing research has confirmed that the ideas regarding anammox metabolism that were derived from genome analyses are essentially correct.

Roads to NO

NO is a key intermediate not only in anammox bacteria, but in many denitrifying microorganisms. In the latter, NO is commonly produced by the reduction of nitrite to NO

(Figure 1, reaction 1) and this reaction is catalyzed by one of the two completely different and well-studied enzymes, heme protein NirS (*cd₁* nitrite reductase), which contains a designated heme variant (heme *d*) as the catalytic center (Box 2) [34-37] or copper-containing NirK [36]. The genome of *K. stuttgartiensis* harbors the heme *c* and heme *d* structural genes including those coding for heme *d* assembly, but these are hardly expressed at the transcriptional and protein levels compared to the ones encoding other key catabolic proteins [27,28,30]. Remarkably, the NirS genes are among the highest expressed ones in the marine anammox species *Scalindua brodae* [39]. In contrast, the *Jettenia caeni* strain KSU-1 is lacking NirS, but possesses NirK instead [40], whereas *Brocadia* species are devoid of known nitrite reductase genes [42,43]. Apparently, anammox bacteria utilize different enzymes to reduce nitrite to NO, which has to include an as-yet elusive nitrite reductase. Based on their catalytic potentials, this new nitrite reductase might be found among the HAO-like proteins described next.

A Collection of HAO-like Multiheme Proteins in Anammox Bacteria

As alluded above, hydrazine oxidation is catalyzed artificially by HAO, a structurally complex homotrimeric protein that binds eight *c*-type hemes per subunit [43,44]. Seven of these hemes are His/His ligated and constitute a wire of heme groups that transfer the (four) electrons derived from hydroxylamine to nitrite (eqn. 2) via different routes to exit heme 1 (Figures 2B-C). Here, the catalytic center is heme 4, which lacks a distal ligand, offering the site for substrate binding and conversion (Figure 2D). A peculiar property of heme 4 is that it makes two covalent bonds with a tyrosine from a neighboring subunit, which binds the subunits to each other. This tyrosine bonding causes the heme 4 structure to become highly ruffled, resulting in a characteristic absorbance band around 460 nm in the UV-Vis spectrum

of the reduced enzyme. Due to this absorbance band, catalytic heme 4 is termed the P₄₆₀ prosthetic group.

Amazingly, anammox genomes contain genes coding for 10-11 different HAO-like octaheme protein paralogs that are highly conserved among the sequenced genomes. Most of these paralogs are expressed to variable degrees in *K. stuttgartiensis* (Figure 2A). The immediate questions then are: which gene product represents the hydrazine-oxidizing enzyme hydrazine dehydrogenase (HDH) and what is the function of the other ones? By direct purification two of these HAO-like proteins, both major proteins in the cells, were functionally identified [28, 45-48]. The first one, kustd1061, named after the *K. stuttgartiensis* (kust) gene identifier, was studied in detail and its crystal structure was resolved (Figures 2B-D) [47]. Despite having only 30% amino acid sequence identity, the structures of kustd1061 and HAO from the aerobic ammonium oxidizer *Nitrosomonas europaea* (NeHAO) are fully superimposable and both proteins share highly similar catalytic sites. Like NeHAO (eqn. 2), kustd1061 oxidizes hydroxylamine, but instead of nitrite its end product is NO (Figure 1, reaction 4). This difference in activity is the result of a small difference near the catalytic site preventing the addition of water to produce nitrite [47]. After its activity, kustd1061 is denoted hydroxylamine oxidase (KsHOX). However, the physiological function of KsHOX would remain enigmatic since hydroxylamine is not an established intermediate in the anammox process. Similar to NeHAO, KsHOX can also oxidize hydrazine to N₂. In crystals of KsHOX soaked with hydrazine, hydrazine (or more likely its two-electron oxidation product diazene (NH=NH)) is seen as being bound to the catalytic heme 4 (Figure 2D) [47]. As is the case for NeHAO, this hydrazine oxidizing activity is observed *in vitro*, but it is not a physiological one. The genuine HDH in *K. stuttgartiensis* is represented by kustc0694 [28,48] and a close homolog of it has been described for *J. caeni* [45]. In the genome of *K. stuttgartiensis* a

second gene (*kustd1340*) with a high sequence identity to HDH (>98% in the translated amino acid sequence) is present. This gene duplication, which is conserved in most genomes of anammox species, most likely represents a second HDH species. The expression of *kustd1340* (on the transcript level) was only detected in *K. stuttgartiensis* cells under stress and its regulation remains to be determined [49].

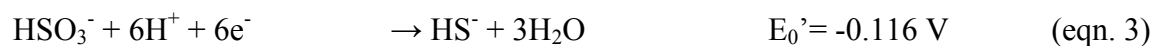
The structural similarity of NeHAO and KsHOX is most likely shared in HDH [48]. All three are homotrimeric proteins with a P₄₆₀ catalytic heme and all three perform oxidative reactions (eqn. 2 and reactions 3 and 4 in Figure 1). Unlike the former two, HDH associates into octamers of trimers ((α_3)₈) carrying an astonishing 192 *c*-type heme molecules [47]. P₄₆₀ seems to be specifically designed to perform oxidative reactions [47,48,50]. The other HAO-like proteins listed in Figure 2A lack the tyrosine involved in covalent bonds with heme 4, and consequently would be devoid of the particular catalytic center, thereby favoring reductive reactions. In *K. stuttgartiensis* cells, we find two notable and quite abundant representatives of these [30,51]. The first one is the *kust0457-0458* protein in which octaheme *kustc0458* and diheme *kustc0457* constitute a heterododecameric ($\alpha_6\beta_6$) complex comprising 60 *c*-type hemes [51]. The second one (*kuste4574*), a close homolog of *kustc0458*, forms part of a novel type of Rieske-heme *b* (R/*b*; *bc*₁) complex (*kuste4569-74*) (Figure 1) [30]. Again, homologs of *kust0457-0458* and of this R/*b* complex, including HAO-like *kuste4574* homologs, are found in all anammox bacteria genomes sequenced thus far. Merely based on protein sequence analyses, it has been postulated that the role of the *kust0457-0458* and *kuste4574* is the reduction of nitrite, making these the elusive nitrite reductases producing NO [29].

Ammonium Production

In the absence of ammonium, anammox bacteria are able to generate this substrate by reducing nitrate or nitrite using electrons derived from the oxidation of supplementary organic or inorganic compounds [18,29]. Nitrite formation from nitrate is mediated by the bidirectional nitrite:nitrate oxidoreductase (NXR) (Figure 1, reaction 5) [51,52]. Under normal (i.e., autotrophic) growth conditions with CO₂ as sole carbon source and with ammonium and nitrite as energy substrates, NXR catalyzes the oxidation of nitrite to nitrate to provide the reducing equivalents for CO₂ fixation. The way nitrite is reduced to ammonium is another missing piece in the anammox puzzle.

In DNRA bacteria, the dissimilatory (respiratory) reduction of nitrite to ammonium (Figure 1, reaction 6) is commonly catalyzed by nitrite reductase NrfA, also referred to as cytochrome *c*₅₅₂ (nitrite reductase) after the absorbance maximum of the catalytic heme [53-56]. NrfA is a dimeric pentaheme protein in which heme 1 represents this catalytic center and hemes 2-5 are involved in electron transfer (Figure 3B). Heme 1 has lysine as the distinctive proximal ligand, which is reflected in the CXXCK binding motif in the protein sequence. Interestingly, these five hemes are fully superimposable to hemes 4-8 in HAO-like octaheme proteins (Figure 3A) and the latter have been suggested to be evolved from the fusion of a NrfA-like progenitor and a triheme protein [50]. In fact, octaheme proteins that catalyze nitrite reduction to ammonium and in which catalytic heme 4 is proximally ligated to a lysine through the CXXCK motif are known from *Thioalkalivibrio* species (Figure 3C) [57-59]. Despite being octaheme proteins, their sequences are unrelated to those of NeHAO and the HAO-like anammox proteins listed in Figure 2A. Still, heme packing is also conserved here (Figure 3). Nitrite reduction to ammonium is also catalyzed by assimilatory nitrite reductases and, at least *in vitro*, by dissimilatory and assimilatory sulfite reductases [60-63]. Reactions catalyzed by

these enzymes, in this case iron sulfur proteins bearing siroheme as their catalytic center (Box 2), are the six-electron reductions of nitrite to ammonium (Figure 1, reaction 6) or sulfite to sulfide (eqn. 3).



These enzymes are somewhat promiscuous in the use of their nitrogenous and sulfurous substrates, which may be the result of the isoelectronic character of the reactions. Siroheme-type sulfite reductase has octaheme counterparts [64-66]. While amino acid sequences of these octaheme sulfite reductases and HAO-like proteins differ completely, the arrangement of their eight hemes are spatially nearly the same (Figure 3D). This conservation applies to one more sulfurous compound-reducing enzyme, octaheme tetrathionate reductase (OTR) from *Shewanella*, which is capable of nitrite reduction as well (Figure 3E) [67,68].

The puzzling aspect is that genomes of anammox bacteria lack all of the above nitrite reductases. However, *Brocadia* [40] and *Jettenia* [41] species have a gene whose translated amino acid sequence fully aligns with pentaheme NrfAs, except that the diagnostic CXXCK motif for the binding of the catalytic heme 1 is replaced by the canonical CXXCH motif. However, this replacement is also seen in genes from established ammonifying bacteria like *Anaerobacter dehalogenans* and *Campylobacter jejuni* [69]. Therefore, the particular pentaheme proteins most likely represent alternative NrfA-like nitrite reductases. Interestingly, the catalytic heme (2) in the *Shewanella* OTR (Figure 3E) has a lysine as its proximal ligand, despite the presence in the amino acid sequence of the CXXCH binding motif [67]. In the crystal structure, the histidine is moved away and the lysine is derived from another part of the protein backbone. Such ligand switch might also hold for alternative NrfA proteins. Nevertheless, the genomes of *Kuenenia* and *Scalindua* species do not contain the alternative *nrfA* gene either. However, they do encode an HAO-like protein (kustd2021),

which is absent in *Brocadia* and *Jettenia*, and consequently might substitute for an ammonium-producing nitrite reductase.

Hydrazine Synthesis

Inspection of the genome of *K. stuttgartiensis*, suggested a unique set of genes (kuste2859-61) to code for HZS [27]. These genes are part of a larger gene cluster (kuste2854-61) (Figure 4A). Direct purification from *K. stuttgartiensis*, in which HZS comprised no less than ~20% of the protein complement, substantiated this prediction and confirmed that the proposed reaction is catalyzed by HZS as shown in Figure 1 (reaction 2) [28]. The recent resolution of the crystal structure provided a clue how HZS might work [70]. Most strikingly, while the overall reaction is completely new, HZS seems to take advantage of the functionality of two well-studied types of enzymes that both deal with hydrogen peroxide: cytochrome *c* peroxidases (CCPs) and catalases.

HZS was crystallized as a crescent-shaped dimer of heterotrimers ($\alpha_2\beta_2\gamma_2$) in which the α , β and γ subunits stand for kuste2861, kuste2860, and kuste2859, respectively (Figures 4B-C). The α subunit is mainly structured by six-bladed β propeller sheets and the β subunit by a seven-bladed β propeller (Figure 4D). Such β propeller architecture is seen in numerous proteins where this structuring provides a solid platform for protein-protein interactions as well as for the binding of cofactors that direct activity in diverse enzymes such as NirS nitrite reductase, NO reductases, methanol- and methylamine dehydrogenases [71,72]. The structure of the γ subunit highly resembles those of CCPs [73,74] and the MauG protein involved in the synthesis of tryptophan tryptophylquinone cofactor of methylamine dehydrogenase [75,76]. As it is the case for CCPs and MauG, the HZS γ subunit contains two *c*-type hemes. The first one, which is His/His ligated, as opposed to the His/Met ligation in CCPs, is located at the

protein surface and the second one, a pentacoordinated heme *c*, is buried inside the protein, representing a catalytic site (Figure 4E). The His/His-ligated heme would then function in electron transfer, as has been established for CCPs [73,74]. The edge-to-edge distances between these two hemes (15 Å) readily permit the rapid transfer of electrons between both. The α subunit is also a diheme protein with a His/His ligated c-type heme near the protein surface and a pentacoordinated heme deep inside (Figure 4E). The distance between both hemes (31 Å) is too large to permit electron electron transfer between these centers at appreciable rate and the function of the His/His-ligated heme is unknown. However, the pentacoordinated heme provides a second catalytic site. A peculiar feature of this heme is that its proximal ligand is not the histidine of the CXXCH heme-binding motif, but a tyrosine of the amino acid backbone. The histidine of the CXXCH heme-binding motif is instead moved away by a zinc atom. Tyrosine ligation is common in many catalases that split hydrogen peroxide into oxygen and water by an intramolecular redox reaction (“disproportionation”) [77,78]. The α and γ subunits are interconnected by a system of tunnels, including one that gives access to the protein surface (Figure 4F). The latter would allow the entry and exit of substrate and product, respectively, whose trafficking is mostly likely directed by a flexible loop derived from the β subunit.

CCPs catalyze the two-electron reduction of hydrogen peroxide to water: $\text{H}_2\text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow 2 \text{H}_2\text{O}$. In analogy, the γ subunit could mediate the three-electron reduction of NO to hydroxylamine, which is isoelectronic with H_2O_2 reduction to water by CCPs. The observation that HZS is able to oxidize hydroxylamine [28], as HOX does (Figure 1, reaction 4), supports the idea of NH_2OH and NO being reactants. The transfer of hydroxylamine to the α subunit catalytic heme and its subsequent combination with ammonia (NH_3), which is associated with the reduction and oxidation of the nitrogen atoms of NH_2OH and NH_3 ,

respectively (a so-called “comproportionation” reaction) then would yield hydrazine (Figure 4F). Ammonia could be supplied via another, minor tunnel in the protein. Intriguingly, this mechanism highly resembles the Raschig process used in industrial hydrazine synthesis. In this process, ammonia is oxidized to chloramine- reflected in HZS in hydroxylamine -or a hydroxylamine-derived amine group ligated to the heme iron- that reacts with a second ammonia to produce hydrazine. A consequence of the anticipated process is that hydroxylamine would have access to the catalytic sites, but also might escape from the protein. Upon its loss, the HOX protein could come into play by neatly regenerating hydroxylamine into NO and three electrons that are required for the hydrazine synthase reaction, resolving the enigmatic question of its physiological role (Figure 1, reaction 4).

The view presented so far is primarily based on what the protein structure tells us. Many details regarding the HZS reaction mechanism remain to be established, which will not be an easy task. As isolated HZS is an extremely slow enzyme (specific activity, 20 nmol h⁻¹ mg protein⁻¹) and well over 90% of the activity is already lost by disrupting the cells [28]. This loss might be due to an inactivation of HZS itself, but also could also be the result of the disruption of a multicomponent enzyme system. Such system might include other gene products found the HZS gene cluster (Figure 4A) as there are a membrane-bound, heme *b*-containing electron transfer module (kuste2855-56) and a soluble triheme protein (kuste2854), potentially involved in the gathering of electrons derived from menaquinol oxidation and their delivery to HZS [29,30] (Figure 1). However, this plunges us into another wide-open field in anammox research, which only has been explored conceptually [29]: the coupling at the anammoxosome membrane between catabolism and the generation of a proton-motive force for ATP synthesis. A small corner of the veil has only been raised very recently by the elucidation of a series of exceptional membrane-bound protein complexes (Figure 1) [30].

Concluding remarks

A survey into the progress in the research on anammox biochemistry, even though in its infancy, leaves us with an amazing view into the way these microorganisms took advantage of common biochemical processes and mechanisms. Enzymic reactions were directed so that they offered anammox bacteria their unique ecological space. The HAO-like octaheme proteins are excellent examples of this. These multiheme proteins are broadly used for the interconversion of nitrogenous and sulfurous compounds. Here, highly diverse octaheme proteins convergently evolved into common structures (Figure 3). In anammox bacteria the opposite might be the case; a divergent evolution may have resulted in different functionalities, most of which are still elusive. Future research, as done for HOX [47] and HDH [48], has to establish the unknown functions and, more importantly, may reveal how each protein is tuned to a specific function by the presence common catalytic sites and conserved, *c*-type heme-based electron transfer pathways. In general, the structure-function relationship is only partially understood regarding multiheme proteins. The anammox HAO-like proteins offer a grand opportunity to address this issue systematically. Hydrazine synthase, the only enzyme besides N₂O-forming NO reductase that is capable of forging an N-N bond, is a second example. Here, two functionalities seem to be combined to make something new: hydrazine. However, the reaction mechanism by which hydrazine is produced, is largely unknown, but it most certainly will involve a delicate interplay and timing of redox reactions and substrate tunneling. Hydrazine is the most powerful reductant in nature. In connection with its oxidation at the anammoxosome a proton-motive force has to be established that drives ATP synthesis (Figure 1 and Box 1). These respiratory processes depend on membrane-bound respiratory systems, including novel *bc*₁-like complexes [30]. Their study merits further attention not only from the bio-energetic point of view but also because genes coding for similar protein complexes are found in the (meta)genomes of many

anaerobic microorganisms that play an often unknown, but possibly crucial role in anaerobic microbial processes.

Anammox bacteria are found as different species and in an enormous range of subspecies. Genomes sequenced thus far indicate that all species rely on a common inventory of enzymes described in this paper, albeit with notable variations. The key intermediate NO and ammonium as a primary substrate appear to be formed from nitrite by different and also unknown nitrite reductases. A differential use and expression of these enzymes may provide a (sub)species its own ecological niche. The reason for this differential use is unclear, but the finding of answers regarding anammox may reveal new concepts as to the way biochemical diversification underlies biological diversity, ultimately reflected in the millions [93,94] or even a trillion [95] of microbial species that are found on earth.

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References

1. Vitousek, P.M. *et al.* (2009) Nutrient imbalances in agricultural development. *Science* 324, 1519-1520
2. Canfield, D.E. *et al.* (2010) The evolution and future of Earth's nitrogen cycle. *Science* 330, 192-196
3. Rockström, J. *et al.* (2009) A safe operating space for humanity. *Nature* 461, 472-475.
4. Kowalchuk, G.A. and Stephen, J.R. (2001) Ammonia-oxidizing bacteria: a model for molecular microbial ecology. *Annu. Rev. Microbiol.* 55, 485-529

5. Stahl, D.A. and de la Torre, J.R. (2012) Physiology and diversity of ammonia-oxidizing archaea. *Annu. Rev. Microbiol.* 66, 83-101
6. Monteiro, M. *et al.* (2014) The history of aerobic ammonia oxidizers: from the first discoveries to today. *J. Microbiol.* 52, 537-547
7. Daims, H. *et al.* (2016) A New Perspective on Microbes Formerly Known as Nitrite-Oxidizing Bacteria. *Trends Microbiol.* doi: 10.1016/j.tim.2016.05.004
8. Kraft, B. *et al.* (2011) Microbial nitrate respiration--genes, enzymes and environmental distribution. *J. Biotechnol.* 155, 104-117
9. Simon, J. and Klotz, M.G. (2013) Diversity and evolution of bioenergetic systems involved in microbial nitrogen compound transformations. *Biochim Biophys Acta* 1827, 114-135
10. Lu, H. *et al.* (2014) Microbial ecology of denitrification in biological wastewater treatment. *Water Res.* 64, 237-254
11. Hooper, A.B. *et al.* (1997) Enzymology of the oxidation of ammonia to nitrite by bacteria. *Antonie Van Leeuwenhoek* 71, 59-67
12. Arp, D.J. *et al.* (2002) Molecular biology and biochemistry of ammonia oxidation by *Nitrosomonas europaea*. *Arch. Microbiol.* 178, 250-255
13. van de Graaf, A.A. *et al.* (1995) Anaerobic oxidation of ammonium is a biologically mediated process. *Appl. Environ. Microbiol.* 61, 1246-1251
14. Strous, M. *et al.* (1998) The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. *Appl. Microbiol. Biotechnol.* 50, 589-596
15. Kartal, B. *et al.* (2011). Cultivation, detection, and ecophysiology of anaerobic ammonium-oxidizing bacteria. *Methods Enzymol.* 486, 89-108

16. Schmid, M.C. *et al.* (2005) Biomarkers for in situ detection of anaerobic ammonium-oxidizing (anammox) bacteria. *Appl. Environ. Microbiol.* 71, 1677-1684
17. Song, B. and Tobias, C.R. (2011) Molecular and stable isotope methods to detect and measure anaerobic ammonium oxidation (anammox) in aquatic ecosystems. *Methods Enzymol.* 496, 63-89
18. Kartal, B. *et al.* (2012) Anammox--growth physiology, cell biology, and metabolism. *Adv. Microb. Physiol.* 60, 211-262
19. Oshiki, M. *et al.* (2015) Ecology and Physiology of Anaerobic Ammonium Oxidizing (anammox) Bacteria. *Environ. Microbiol.* doi: 10.1111/1462-2920.13134
20. Lam, P. and Kuypers, M.M.M. (2011) Microbial nitrogen cycling processes in oxygen minimum zones. *Annu. Rev. Mar. Sci.* 3, 317-345.
21. Devol, A.H. (2015) Denitrification, Anammox, and N₂ production in marine sediments. *Annu. Rev. Mar. Sci.* 7, 403-423.
22. Kartal, B. *et al.* (2010) Sewage treatment with Anammox. *Science* 328, 702-703
23. Fuerst, J.A. (2005) Intracellular compartmentation in planctomycetes. *Annu. Rev. Microbiol.* 59, 299-328
24. Fuerst, J.A. and Sagulenko, E. (2013) Nested bacterial boxes: nuclear and other intracellular compartments in planctomycetes. *J. Mol. Microbiol. Biotechnol.* 23, 95-103
25. van de Graaf, A.A. *et al.* (1997) Metabolic pathway of anaerobic ammonium oxidation on basis of N-15 studies in a fluidized bed reactor. *Microbiology* 143, 2415-2421
26. Schalk, J. *et al.* (1998) The anaerobic oxidation of hydrazine: a novel reaction in microbial nitrogen metabolism. *FEMS Microbiol. Lett.* 158, 61-67
27. Strous, M. *et al.* (2006) Deciphering the evolution and metabolism of an anammox bacterium from a community genome. *Nature* 440, 790-794

28. Kartal, B. *et al.* (2011) Molecular mechanism of anaerobic ammonium oxidation. *Nature* 479, 127–130
29. Kartal, B. *et al.* (2013) How to make a living from anaerobic ammonium oxidation. *FEMS Microbiol. Rev.* 37, 428-461
30. de Almeida, N.M. *et al.* (2016) Membrane-bound electron transport systems of an anammox bacterium: a complexome analysis. *Biochim. Biophys. Acta* 1857, 1694-1704
31. Hooper, A B. *et al.* (1997) Enzymology of the oxidation of ammonia to nitrite by bacteria. *Antonie Van Leeuwenhoek* 71, 59-67
32. Hooper, A.B. and Nason A. (1965) Characterization of hydroxylamine-cytochrome *c* reductase from the chemoautotrophs *Nitrosomonas europaea* and *Nitrosocystis oceanus*. *J. Biol. Chem.* 240, 4044-4057
33. Terry, K.R. and Hooper, A.B. (1981) Hydroxylamine oxidoreductase: a 20-heme, 200 000 molecular weight cytochrome *c* with unusual denaturation properties which forms a 63 000 molecular weight monomer after heme removal. *Biochemistry* 20, 7026-7032
34. Jafferji, A. *et al.* (2000) X-ray crystallographic study of cyanide binding provides insights into the structure-function relationship for cytochrome *cd₁* nitrite reductase from *Paracoccus pantotrophus*. *J. Biol. Chem.* 275, 25089-25094
35. Ferguson, S.J. and Fülöp, V. (2000) Cytochrome *cd₁* nitrite reductase structure raises interesting mechanistic questions. *Subcell. Biochem.* 35, 519-540
36. Allen, J.W. *et al.* (2005) Why isn't 'standard' heme good enough for *c*-type and *d₁*-type cytochromes? *Dalton Trans.* 7, 3410-3418
37. Rinaldo, S. *et al.* (2008) New insights into the activity of *Pseudomonas aeruginosa cd₁* nitrite reductase. *Biochem. Soc. Trans.* 36, 1155-1159
38. Nojiri, M. *et al.* (2007) Structure and function of a hexameric copper-containing nitrite reductase. *Proc. Natl. Acad. Sci. U.S.A.* 104, 4315-4320

39. van de Vossenberg J. *et al.* (2013) The metagenome of the marine anammox bacterium '*Candidatus Scalindua profunda*' illustrates the versatility of this globally important nitrogen cycle bacterium. *Environ. Microbiol.* 15, 1275-1289
40. Hira, D. *et al.* (2012) Anammox organism KSU-1 expresses a NirK-type copper-containing nitrite reductase instead of a NirS-type with cytochrome *cd*₁. *FEBS Lett.* 586, 1658-1663
41. Oshiki, M. *et al.* (2015) Draft genome sequence of an anaerobic ammonium-oxidizing bacterium, "*Candidatus Brocadia sinica*". *Genome Announc.* 3, e00267-15.
42. Speth, D.R. *et al.* (2016) Genome-based microbial ecology of anammox granules in a full-scale wastewater treatment system. *Nat. Commun.* 7, 11172.
43. Igarashi, N. *et al.* (1997) The 2.8 Å structure of hydroxylamine oxidoreductase from a nitrifying chemoautotrophic bacterium, *Nitrosomonas europaea*. *Nat. Struct. Biol.* 4, 276-284
44. Cedervall, P. *et al.* (2013) Structural studies of hydroxylamine oxidoreductase reveal a unique heme cofactor and a previously unidentified interaction partner. *Biochemistry* 52, 6211-6218
45. Shimamura, M. *et al.* (2007) Isolation of a multiheme protein with features of a hydrazine-oxidizing enzyme from an anaerobic ammonium-oxidizing enrichment culture. *Appl. Environ. Microbiol.* 73, 1065-1072
46. Shimamura, M. *et al.* (2008) Another multiheme protein, hydroxylamine oxidoreductase, abundantly produced in an anammox bacterium besides the hydrazine-oxidizing enzyme. *J. Biosci. Bioeng.* 105, 243-248
47. Maalcke, W.J. *et al.* (2014) Structural basis of biological NO generation by octaheme oxidoreductases. *J. Biol. Chem.* 289, 1228-1242

48. Maalcke, W.J. *et al.* (2016) Characterization of anammox hydrazine dehydrogenase, a key N₂-producing enzyme in the global nitrogen cycle. *J. Biol. Chem.* 291, 17077-17092
49. Hu, Z. *et al.* (2013) Lysozyme and penicillin inhibit the growth of anaerobic ammonium-oxidizing planctomycetes. *Appl. Environ. Microbiol.* 79, 7763-7769
50. Klotz, M.G. *et al.* (2008) Evolution of an octahaem cytochrome *c* protein family that is key to aerobic and anaerobic ammonia oxidation by bacteria. *Environ. Microbiol.* 10, 3150-3163
51. de Almeida, N.M. *et al.* (2015) Immunogold Localization of Key Metabolic Enzymes in the Anammoxosome and on the Tubule-Like Structures of *Kuenenia stuttgartiensis*. *J. Bacteriol.* 197, 2432-2441
52. de Almeida, N.M. *et al.* (2011) Proteins and protein complexes involved in the biochemical reactions of anaerobic ammonium-oxidizing bacteria. *Biochem. Soc. Trans.* 39, 303-308
53. Einsle, O. *et al.* (2002) Mechanism of the six-electron reduction of nitrite to ammonia by cytochrome *c* nitrite reductase. *J. Am. Chem. Soc.* 124, 11737-11745
54. Lockwood, C.W. *et al.* (2011) Characterization of the active site and calcium binding in cytochrome *c* nitrite reductases. *Biochem. Soc. Trans.* 39, 1871-1875
55. Einsle, O. (2011) Structure and function of formate-dependent cytochrome *c* nitrite reductase, NrfA. *Methods Enzymol.* 496, 399-422
56. Simon, J. and Kroneck, P.M. (2014) The production of ammonia by multiheme cytochromes C. *Met. Ions Life Sci.* 14, 211-236
57. Polyakov, K.M. *et al.* (2009) High-resolution structural analysis of a novel octaheme cytochrome *c* nitrite reductase from the haloalkaliphilic bacterium *Thioalkalivibrio nitratireducens*. *J. Mol. Biol.* 389, 846-862

58. Tikhonova, T. *et al.* (2012) Comparative structural and functional analysis of two octaheme nitrite reductases from closely related *Thioalkalivibrio* species. *FEBS J.* 279, 4052-4061
59. Doyle, R.M. *et al.* (2013) Contrasting catalytic profiles of multiheme nitrite reductases containing CxxCK heme-binding motifs. *J. Biol. Inorg. Chem.* 18, 655-667
60. Lin, J.T. and Stewart, V. (1998) Nitrate assimilation by bacteria. *Adv. Microb. Physiol.* 39, 1-30
61. Crane, B.R. *et al.* (1995) Sulfite reductase structure at 1.6 Å: evolution and catalysis for reduction of inorganic anions. *Science* 270, 59-67
62. Crane, B.R. and Getzoff, E.D. (1996) The relationship between structure and function for the sulfite reductases. *Curr. Opin. Struct. Biol.* 6, 744-756
63. Simon, J. and Kroneck, P.M. (2013) Microbial sulfite respiration. *Adv. Microb. Physiol.* 62, 45-117
64. Kern, M. *et al.* (2011) The *Wolinella succinognes* *mcc* gene cluster encodes an unconventional respiratory sulphite reduction system. *Mol. Microbiol.* 82, 1515-1530
65. Shirodkar, S. *et al.* (2011) The octahaem SirA catalyses dissimilatory sulfite reduction in *Shewanella oneidensis* MR-1. *Environ. Microbiol.* 13, 108-115
66. Hermann, B. *et al.* (2015) The octahaem MccA is a haem c-copper sulfite reductase. *Nature* 520, 706-709
67. Mowat, C.G. *et al.* (2004) Octaheme tetrathionate reductase is a respiratory enzyme with novel heme ligation. *Nat. Struct. Mol. Biol.* 11, 1023-1024
68. Atkinson, S.J. *et al.* (2007) An octaheme c-type cytochrome from *Shewanella oneidensis* can reduce nitrite and hydroxylamine. *FEBS Lett.* 581, 3805-3808
69. Welsh, A. *et al.* (2014) Refined NrfA phylogeny improves PCR-based *nrfA* gene detection. *Appl. Environ. Microbiol.* 80, 2110-2119

70. Dietl, A. *et al.* (2015) The inner workings of the hydrazine synthase multiprotein complex. *Nature* 527, 394-397
71. Chen, C.K. *et al.* (2011) The many blades of the β -propeller proteins: conserved but versatile. *Trends Biochem. Sci.* 36, 553-561
72. Xu, C. and Min, J. (2011) Structure and function of WD40 domain proteins. *Protein Cell* 2, 202-214
73. Pettigrew, G.W. *et al.* (2006) Structure and mechanism in the bacterial dihaem cytochrome *c* peroxidases. *J. Inorg. Biochem.* 100, 551-567
74. Poulos, T.L. (2010) Thirty years of heme peroxidase structural biology. *Arch. Biochem. Biophys.* 500, 3-12
75. Wilmot, C.M. and Yukl, E.T. (2013) MauG: a di-heme enzyme required for methylamine dehydrogenase maturation. *Dalton Trans.* 42, 3127-3135
76. Shin, S. and Davidson, V.L. (2014) MauG, a diheme enzyme that catalyzes tryptophan tryptophylquinone biosynthesis by remote catalysis. *Arch. Biochem. Biophys.* 544, 112-118
77. Zamocky, M. *et al.* (2008) Evolution of catalases from bacteria to humans. *Antioxid. Redox Signal.* 10, 1527-1548
78. Alfonso-Prieto, M. *et al.* (2012) The reaction mechanisms of heme catalases: an atomistic view by ab initio molecular dynamics. *Arch. Biochem. Biophys.* 525, 121-130
79. Einsle, O. *et al.* (1999) Structure of cytochrome *c* nitrite reductase. *Nature* 400, 476-480
80. van Niftrik, L. and Jetten, M.S. (2012) Anaerobic ammonium-oxidizing bacteria: unique microorganisms with exceptional properties. *Microbiol. Mol. Biol. Rev.* 76, 585-596
81. van Teeseling, M.C. *et al.* (2014) A new addition to the cell plan of anammox bacteria: "*Candidatus* Kuenenia stuttgartiensis" has a protein surface layer as the outermost layer of the cell. *J. Bacteriol.* 196, 80-89

82. van Teeseling, M.C. *et al.* (2015) Anammox Planctomycetes have a peptidoglycan cell wall. *Nat. Commun.* 6,6878
83. van Niftrik, L.A. *et al.* (2004) The anammoxosome: an intracytoplasmic compartment in anammox bacteria. *FEMS Microbiol. Lett.* 233, 7-13
84. Neumann, S. *et al.* (2014) Isolation and characterization of a prokaryotic cell organelle from the anammox bacterium *Kuenenia stuttgartiensis*. *Mol. Microbiol.* 94, 794-802
85. Sinninghe Damsté, J.S. *et al.* (2002) Linearly concatenated cyclobutane lipids form a dense bacterial membrane. *Nature* 419, 708-712
86. Sinninghe Damsté, J.S. *et al.* (2005) Structural identification of ladderane and other membrane lipids of planctomycetes capable of anaerobic ammonium oxidation (anammox). *FEBS J.* 272, 4270-4283
87. Poulos, T.L. (2014) Heme enzyme structure and function. *Chem Rev.* 114, 3919-3962
88. Kleingardner, J.G. and Bren, K.L. (2015) Biological significance and applications of heme *c* proteins and peptides. *Acc. Chem. Res.* 48, 1845-1852
89. Bewley, K.D. *et al.* (2013) Multi-heme proteins: nature's electronic multi-purpose tool. *Biochim. Biophys. Acta* 1827, 938-948
90. Safarian, S. *et al.* (2016) Structure of a *bd* oxidase indicates similar mechanisms for membrane-integrated oxygen reductases. *Science* 352, 583-586
91. Breuer, M. *et al.* (2014) Electron flow in multiheme bacterial cytochromes is a balancing act between heme electronic interaction and redox potentials. *Proc. Natl. Acad. Sci. U.S.A.* 111, 611-616
92. White, G.F. *et al.* (2016) Mechanisms of Bacterial Extracellular Electron Exchange. *Adv. Microb. Physiol.* 68, 87-138
93. Schloss, P.D. *et al.* (2016) Status of the Archaeal and Bacterial Census: an Update. *MBio.* 7. pii: e00201-16

94. Amann, R. and Rosselló-Móra, R. (2016) After All, Only Millions? *MBio* 7. pii: e00999-16
95. Locey, K.J. and Lennon, J.T. (2016) Scaling laws predict global microbial diversity. *Proc. Natl. Acad. Sci. U.S.A.* 113, 5970-5975

Legends to the Figures

Figure 1. Current view of the anammox metabolism and the role of heme proteins herein. Substrates (ammonium, nitrite) and product (N_2) are highlighted light blue. Reactions are numbered (black circles with white numbers) as described in the text and are catalyzed by the following enzymes (kust numbers in parentheses refer to the gene identifiers of *Kuenenia stuttgartiensis*): 1. Nir, nitrite reductase, in anammox bacteria represented by heme *d* containing NirS, copper-containing NirK, or still to be identified new enzyme species. 2. HZS, hydrazine synthase The alpha, beta and gamma subunits are gene products of kuste2861, kuste2860 and kuste2859, respectively 3. HDH, hydrazine dehydrogenase (kustc0694, kustd1340) 4. HOX, hydroxylamine oxidase (kustd1061) 5. NXR, nitrite:nitrate oxidoreductase comprising the alpha (kustd1700), beta (kustd1703) and gamma (kustd1704) subunits [30,51,52] 6. Nrf, nitrite reductase forming ammonium, which is represented in anammox bacteria by a variant of NrfA or by a novel enzyme. Note that reactions occur in the anammoxosome (Box 1). Substrate and electron flows are indicated by black and red arrows, respectively. Dashed lines denote reactions and processes that have to be established. Standard midpoint redox potentials at pH 7 (E_0') of redox reactions are indicated in parentheses and the number of electrons involved in these reactions are mentioned in the red diamonds that represent yet to be identified electron carriers (*c*-type hemes, blue copper proteins) that shuttle the electrons between the different enzymes. Heme *b*, *c*-type octaheme and other *c*-type multiheme proteins are marked red, purple and orange, respectively. The (four) electrons derived from hydrazine oxidation (reaction 3) are branched to two novel Rieske-heme *b* complexes (*R/b*; *bc*₁ complexes), *R/b*-2 (kustd1480-85) and *R/b*-3 (kuste4569-74); the third complex, *R/b*-1 (kuste3096-97) is only lowly expressed and is not shown [30]. These *R/b* complexes may have a crucial but poorly understood complex role in energy metabolism as there are the reduction of menaquinone-7 (MQ), pumping of protons (“ H^+ ”)

across the anammoxosome membrane to create the proton-motive force that drives ATP synthesis by the ATP synthase (ATPase), and the reduction of NAD(P)^+ to NAD(P)H at one of the subunits that form part of the complexes [30]. *R/b-3* also might catalyze the reduction of nitrite to NO by an associated octaheme protein (kuste4574). The reduction of NAD(P)^+ withdraws electrons from the cyclic electron flow, which have to be replenished by the oxidation of nitrite to nitrate by NXR (reaction 5). The three electrons needed for hydrazine synthesis (reaction 2) are thought to be provided by the oxidation of reduced menaquinone (MQH_2), which is catalyzed by an electron transfer module (ETM) composed of heme *b*-containing kuste2856 and the heptaheme protein kuste2855 [30].

Figure 2. Hydroxylamine oxidoreductase (HAO)-related octaheme proteins in *Kuenenia stuttgartiensis*. (A) HAO-like proteins in the *Kuenenia stuttgartiensis* (kust) genome. Highly homologous proteins are boxed by dashed lines. Lengths of the polypeptides are drawn to scale (aa, amino acids) and homologous cytochrome *c*-rich parts are vertically aligned. Redox partners represent (potential) electron transfer companions found in the same gene cluster. Expression values are given as n-fold coverage of Solexa RNA sequencing of the *K. stuttgartiensis* transcriptome [27,28]. Structural motifs: TMH, transmembrane-spanning helix; cleavage site, N-terminal cleavage site; multi-copper oxidase, multicopper oxidase domain; Catalysis, catalytic heme; CXXCH, heme *c*-binding motif; CXXXXCH, unusual heme *c*-binding motif in hydrazine dehydrogenases (kustc0694 and kustc1340); Tyrosine, tyrosine covalently linking subunits; laminin, laminin sequence. It should be noted that these HAO-like proteins are conserved in all sequenced genomes of anammox bacteria, except for kustd2021. (B) Overall architecture of the homotrimeric hydroxylamine oxidase from *Kuenenia stuttgartiensis* (KsHOX) (PDB ID code 4N4L) [47]. Its three subunits are displayed in different colors. The localization of the catalytic site is indicated by the square. (C) Arrangement of the *c*-type hemes in homotrimeric hydroxylamine oxidase from *K. stuttgartiensis* (KsHOX). The Figure shows the outline of the structure of KsHOX (PDB ID code 4N4J) seen from the bottom along the 3-fold symmetry axis featuring the 24 hemes. The

8 hemes present in the same monomer have the same color and are numbered as indicated. Note that hemes are arranged in a ring-like structure. Arrows indicate the routes electrons can take to exit heme 1 following substrate oxidation at the catalytic site (heme 4). The spatial arrangement of these hemes is fully conserved in hydroxylamine oxidoreductase from *N. europaea* (NeHAO) [43,44,47]. (D) X-ray structure of the heme 4 (P₄₆₀) catalytic center of KsHOX soaked with hydrazine seen from the same axis as in (B). A dinitrogen species, a putative diazene (HN=NH) (blue) is seen on top of the heme. Structural images were made using PyMOL (<http://www.pymol.org>). Panel A was adapted from [29] and panels BCD were adapted from [48].

Figure 3. Structural features of multiheme proteins involved in the conversion of nitrogen and sulfur compounds. Structures of the monomers that are rainbow-colored in going from the N- to the C-terminal protein sequences are shown on the left, heme arrangements with the central iron atoms colored orange are in the middle and quaternary structures with one of the subunits in blue are displayed on the right. Hemes are numbered in the order as found in the protein sequence. Catalytic hemes are circled in red. Hemes that constitute the electron entry or exit points are circled blue. Hemes circled green are at the contact site with a neighboring subunit, enabling electron wiring in between different subunits as shown in Figure 2C. Note the conservation in heme spatial arrangements despite the substantial divergence in overall protein structures (and protein sequences). (A) Homotrimeric HAO-like hydroxylamine oxidase from *Kuenenia stuttgartiensis* (KsHOX; PDB ID code 4N4J) [45] also shown in Figures 2B-D. (B) Ammonium-forming homodimeric pentaheme nitrite reductase (NrfA) from *Sulfurospirillum deleyianum*, PDB ID code 1QDB [53,79]. (C) Hexameric octaheme nitrite reductase (ONR) from *Thioalkalivibrio nitratireducens* (PDB ID code 2OT4) [57]. (D) Homotrimeric octaheme sulfite reductase MccA from *Wolinella*

succinogenes (PDB ID code 4RKM). In this protein, electron-entry heme 8 is bound by the non-canonical (CX₁₅CH) binding motif, which gives this heme a different orientation, possibly facilitating the better interaction with the putative electron donor, iron-sulfur protein MccC [66]. (E) Monomeric octaheme tetrathionate reductase from *Shewanella oneidensis* (PDB code 1SP3) [67]. This protein also performs reduction of nitrite to ammonium [68]. Modified from [66] with permission.

Figure 4. Gene cluster organization and structural properties of the hydrazine synthase (HZS) system from *Kuenenia stuttgartiensis*. (A) Structural organization of its gene products. Lengths of the gene products and the position of structural motifs are drawn to scale (aa, amino acids). Structural motifs are specified in the Figure. Numbers refer to the kuste gene numbers. Abbreviations: cleavage site, N-terminal cleavage site; TMH, transmembrane-spanning helix. (B) Overall structure of the HZS complex; α subunits are colored green, β subunits are blue, and γ subunits are grey (PDB ID code 5C2V) [70]). (C) Surface representation highlighting the heme (sticks) and metal cofactors; Ca, calcium, Zn, zinc. Edge-to-edge distances between the hemes within a subunit are indicated in Ångströms. Subunits are colored as in (B). (D) Structure of the seven-bladed propeller β subunit. (E) Structure of the catalytic heme (γ II) of the γ subunit. Note that this heme is covalently bound to protein by three thioether bonds, two (γ Cys102 and γ Cys105) derived from the canonical CXXCH heme-binding motif and a unique binding to the C1 porphyrin methyl derived from γ Cys165. The heme iron binds water (red sphere) as its upper (distal) ligand. (F) Structure of the catalytic heme (α I) of subunit α . Note that this heme has a tyrosine (α Tyr591) as its proximal ligand. The histidine (α His587) of the CXXCH binding motif is moved away by a zinc atom (blue sphere) that also coordinated with one of the heme propionyl groups, with a water molecule (red sphere) and with a cysteine (α Cys303). (G) Cartoon showing the

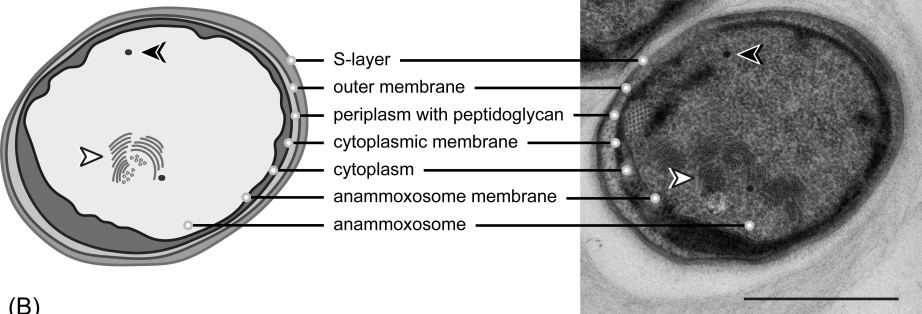
proposed reaction mechanism (see text for further details) as suggested by the crystal structure of HZS. Structural images were made using PyMOL (<http://www.pymol.org>). Panel A was adapted from [29], and panels B, C and G were adapted from [70].

Box 1. Cell Plan of Anammox Bacteria and the Anammoxosome

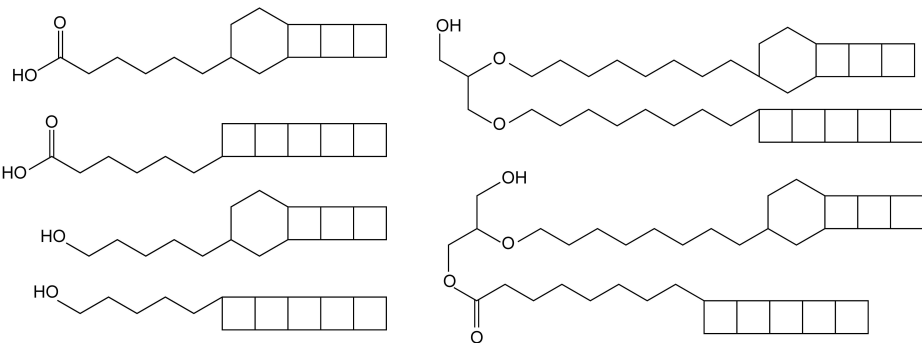
Contrary to most Prokaryotes, anammox bacteria have a rather complicated cell plan (Figure IA) [80]. In essence, their cell plan consists of three membrane systems. The outermost membrane together with a thin peptidoglycan layer constitutes the cell wall, which may be covered by S-layer protein lattice [81,82]. The second membrane layer surrounds the cytoplasm, leaving a periplasmic space in between these two outer membrane systems like in Gram-negative bacteria. The nucleoid (DNA), transcription, translation and household machinery as well as anabolic enzymes are present in the cytoplasm. The largest part of the cell is comprised of a vacuolar cell organelle, the anammoxosome, which is fully enclosed by the third membrane layer [83,84]. Within this organelle, the enzymes involved in catabolism, such as the HAO-like proteins and HZS, discussed in this paper are found; the nitrite-nitrate oxidoreductase (NXR) system is typically associated with tubule-like structures that cross the anammoxosome [51]. An unusual feature of anammox cell membranes is their composition (Figure IB). Herein, C17-C20 saturated fatty acids and alcohols are fused by *cis*-junctions to make ladder-like ('ladderane') cyclobutane and cyclohexane ring systems [85,86].

Figure I. Cell Plan and Lipid Structures of Anammox Bacteria. (A) Schematic overview (left) and transmission electron microscopy image (right) of a cell of the anammox bacterium *Kuenenia stuttgartiensis*. Within the central organelle, the anammoxosome, tubule-like structures are found (white arrow heads) as well as electron-dense iron-rich particles (black arrowheads). Note that not all tubule-like structures seen in the micrograph are represented in the schematic overview. Scale bar, 500 nm. (B) C17-C20 ladderane lipids containing 3-5

(A)



(B)



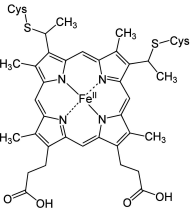
cyclobutane rings, with or without a cyclohexane ring. Fatty acids are esterified with methanol or the glycerol backbone, and alcohols are ether-linked to glycerol, all in different combinations. Adapted with permission from [49, 50].

Box 2. Heme proteins

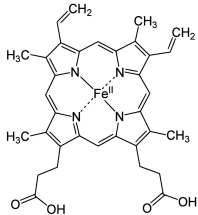
The chemical properties offered by iron protoporphyrins IX (heme, Fig. 1) are exploited in numerous proteins that perform a wide array of functions, including electron transfer, redox catalysis, gas sensing and gas transport [36,87-89]. In these proteins, heme may be present as one of a number of derivatives, most notably heme *c* and heme *b*. By structural modifications, the reactivity of the heme can be directed. Heme *d*, for instance, is the catalytic cofactor of nitrite reductases producing NO [34-37] and of *bd* terminal oxidase [90], while siroheme is the catalytic center of assimilatory nitrite reductases as well as assimilatory and dissimilatory sulfite reductases, performing the 6-electron reduction of their substrates [60-63]. Heme *c* is a ubiquitous constituent of redox proteins [87-89]. Unlike the other derivatives, heme *c* is covalently bound to the protein backbone by two cysteine (C) thioethers, which is commonly seen in a protein sequence by the CXXCH binding motif (X stands for any amino acid). In this binding, the histidine (H) constitutes the fifth, so-called proximal, ligand to the iron heme. The sixth (“distal”) ligand position may be vacant, providing a site for substrate binding and/or redox catalysis. The distal ligand position also may be taken by a protein amino acid, usually a histidine (His/His ligation) or methionine (His/Met ligation), making the heme an electron-transferring or electron-storing one [88,89]. By a combination of enzyme-structural factors, the oxidation-reduction potential of a *c*-type heme can be precisely poised over a range of more than 800 mV [88,91]. Multiheme proteins possess anywhere from two to more than 20 *c*-type hemes, which are spatially arranged with respect to another such that electrons can be transferred over a long range or stored in order to fine-tune reaction rates [88,91,92].

Structures of Heme Molecules.

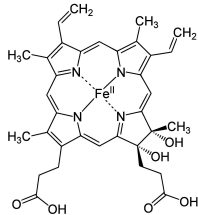
heme c



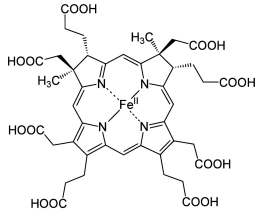
heme b



heme d



siroheme



What are the specific functions of different HAO-like proteins and how are they tuned for a specific function?

Which proteins reduce nitrite to NO and ammonium in different anammox species?

What is the catalytic mechanism of hydrazine synthase?

How are proton-/ion-motive forces generated in the anammoxosome?

How do different anammox species find their specific ecological niche using a common inventory of heme proteins?

About 30-70% of all nitrogen that is released into the atmosphere is produced by microorganisms that have been considered impossible for a long-time, the anaerobic ammonium-oxidizing (anammox) bacteria.

Anammox bacteria oxidize ammonium in the absence of oxygen with nitrite as the terminal electron acceptor. Substrate conversion proceeds through two highly toxic intermediates, nitric oxide and hydrazine.

Anammox metabolism relies on multiheme proteins that structurally resemble ones known from other organisms, but that have new functions in the anammox bacteria.

Anammox metabolism resides in a special and unique cell organelle, the anammoxosome. Here, energy released in the anammox reaction is used to generate proton-motive force that drives ATP synthesis. This respiratory process is supported by novel membrane-bound protein complexes.

