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Review article

Bacterial nitrate reductases: Molecular and biological aspects of nitrate reduction

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Abstract

Nitrogen is a vital component in living organisms as it participates in the making of essential biomolecules such as proteins, nucleic acids, etc. In the biosphere, nitrogen cycles between the oxidation states +V and −III producing many species that constitute the biogeochemical cycle of nitrogen. All reductive branches of this cycle involve the conversion of nitrate to nitrite, which is catalyzed by the enzyme nitrate reductase. The characterization of nitrate reductases from prokaryotic organisms has allowed us to gain considerable information on the molecular basis of nitrate reduction. Prokaryotic nitrate reductases are mononuclear Mo-containing enzymes subgrouped as respiratory nitrate reductases, periplasmic nitrate reductases and assimilatory nitrate reductases. We review here the biological and molecular properties of these three enzymes along with their gene organization and expression, which are necessary to understand the biological processes involved in nitrate reduction.

Keywords: Nitrogen cycle; Mononuclear Mo-containing enzymes; Respiratory nitrate reductase; Periplasmic nitrate reductase; Assimilatory nitrate reductase

1. Introduction

Nitrogen is a vital component in essential biomolecules such as proteins, nucleic acids, etc. In the biosphere, nitrogen cycles between the oxidation states +V and −III producing many species that constitute the biogeochemical cycle of nitrogen (N-cycle). This cycle involves a number of redox reactions in which prokaryotes play the main role since only they have the enzymes carrying out these processes [1].

The N-cycle involves a number of redox processes, which are shown schematically in Fig. 1. These processes are completely independent in some cases but share some steps in others. The dissimilatory processes involve the conversion of nitrate into N_2 (respiration) or into ammonia (respiration/ammonification) [1–5]. Respiration is performed by a group of enzymes (Fig. 1) used by the cell to generate the proton motive force (PMF) across the cytoplasmic membrane [6]. Dissimilatory ammonification is also started with the reduction of nitrate to nitrite, but then nitrite is reduced to ammonia. Both respiration and ammonification are energy conserving and can be used as an electron sink. In addition ammonification may play an important role in cell detoxification. The assimilatory process, which also involves the conversion of nitrate to ammonia; starts with the reduction of nitrate in the cytoplasm and is used by the cell to incorporate nitrogen into biomolecules [1,5,7–9]. The process called nitrification is

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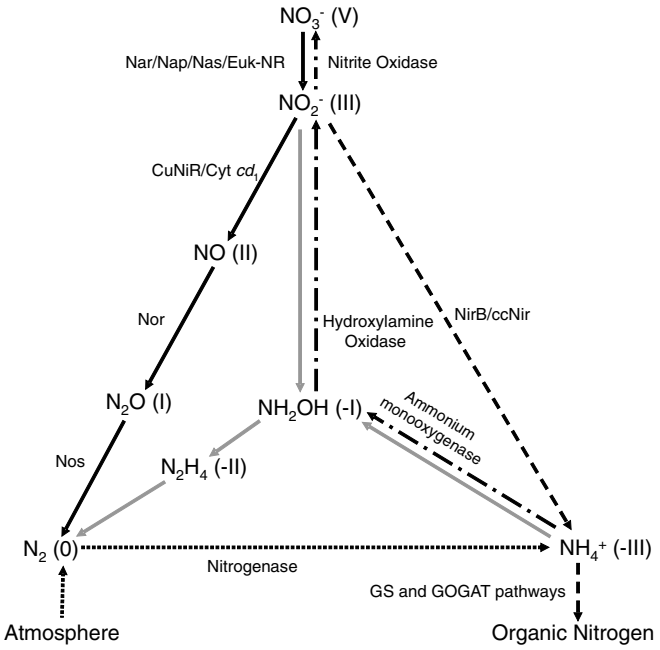

Fig. 1. The inorganic nitrogen cycle including the enzymes responsible for each step. The oxidation state of each compound is indicated between parentheses. The pathways are identified as follow: black solid line, respiratory pathway (denitrification); dashed line, dissimilatory and assimilatory ammonification (note that nitrate reduction is indicated only as solid arrow); dotted line, nitrogen fixation; dash-dot line, nitrification; grey solid line, ANAMOX.

the only one that involves oxidative reactions, and is attributed exclusively to bacteria from the *Nitrosomonas* and *Nitrobacter* genus [10,11]. The N-cycle is completed with nitrogen fixation, in which the enzyme nitrogenase reduces nitrogen from both the atmosphere and respiration to ammonia [9]. A fifth less characterized process named ANAMOX (anaerobic ammonium oxidation), which involves both oxidative and reductive reactions, is used for bacteria to grow in chemolithoautotrophic conditions using ammonia as electron donor and nitrite as electron acceptor [1,12,13].

All the reductive branches of the N-cycle involve the conversion of nitrate to nitrite. This function is performed by distinct enzymes that catalyze the unique reaction

$$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O \quad E^0 = 420\ \text{mV}$$

Nitrate reductases have been classified by taking into consideration source, localization of the enzyme in the cell, molecular properties of the catalytic center, and function. Several nitrate reductases have been obtained from both prokaryotic and eukaryotic organisms. All of them are mononuclear Mo-containing enzymes that, according to the Hille's classification [14], belong to the dimethyl sulfoxide (DMSO) reductase family with the only exception being eukaryotic nitrate reductases, which belong to the sulfite oxidase (SO) family (Fig. 2). The prokaryotic enzymes can also be sub-grouped as respiratory nitrate reductase (Nar), periplasmic nitrate reductases [15] and assimilatory nitrate reductases [16,17]. Their characterization has allowed us to gain considerable information on the molecular basis of nitrate reduction involved in all the branches of the N-cycle. We review here the biochemical and molecular properties of these three types of enzyme along with their gene organization and expression controls, which

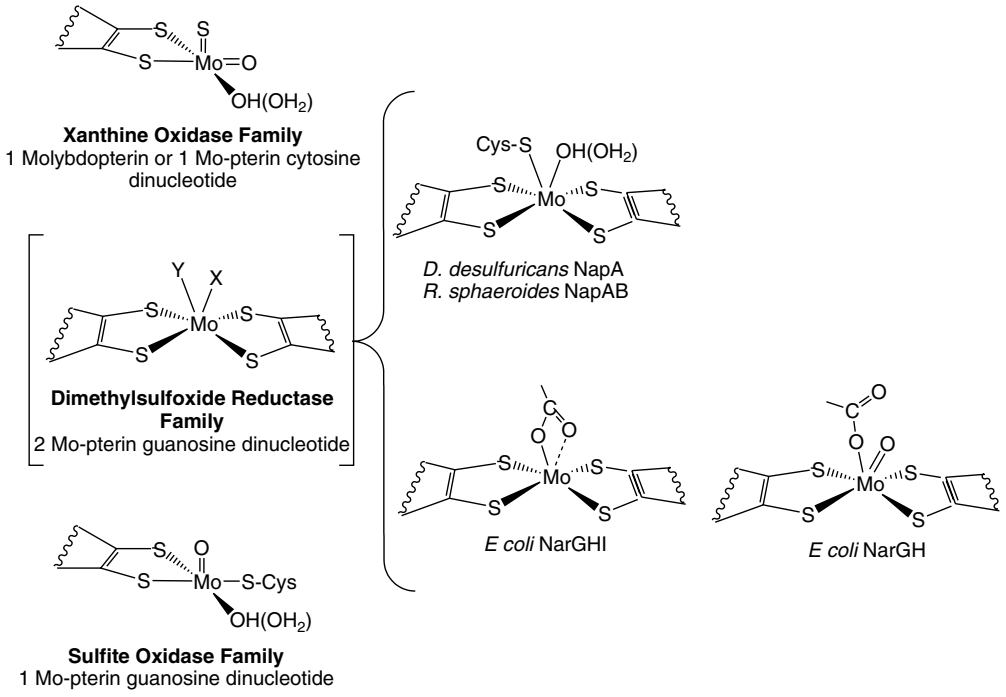

Fig. 2. Left: Active site structure of the three families of mononuclear of mononuclear Mo-containing enzymes. Right: Active site structure of the periplasmic *Dd* NapA and *Rs* NapAB enzymes, and membrane-bound *Ec* NarGH and *Ec* NarGHI. X and Y represent ligands such as oxygen (oxo, hydroxo, water, serine, aspartate), sulfur (cysteine) and selenium (seleniumcysteine) found in the dimethylsulfoxide reductase family.

are necessary to understand the biological processes involved in nitrate reduction.

2. Respiratory nitrate reductases

2.1. Molecular and spectroscopic properties

The Nar enzyme has been isolated from many nitrate-respiring and denitrifying bacteria [18–24]. All the Nars isolated so far are heterotrimeric enzymes composed of the subunits NarG (112–140 kDa), NarH (52–64 kDa) and NarI (19–25 kDa). NarG and NarH are situated in the cytoplasm anchored to the membrane through NarI (Fig. 3). The crystal structures of the oxidized forms of NarGH [25] and NarGHI [26] were reported at resolutions of 2.0 and 1.9 Å, respectively. NarGHI has a flower-like arrangement with dimensions of $90 \times 128 \times 70$ Å^3. The NarI subunit, which is completely immersed in the membrane, is associated with the NarGH dimer through a hydrophobic patch present in NarH. The global arrangement of the catalytic subunit NarG is similar to those from other enzymes belonging to the DMSO reductase family [27], having four domains with α–β type folding. In this sense, it presents high homology with *Dd* Nap, *Ec* Fdh-N, *Ec* Fdh-H and *Dg* Fdh [25–27]. As shown in Fig. 3, NarG contains the active site, a Mo-*bis*MGD cofactor, and an iron–sulfur center of the [4Fe–4S] type (FS0). NarH contains one [3Fe–4S] (FeS4) and three [4Fe–4S] clusters (FeS1–3), whereas NarI contains two *b*-type hemes responsible for the MQH_2 oxidation and proton translocation. These eight redox centers are separated by 12–14 Å giving an electron transfer pathway of about 90 Å. Furthermore, all these metal cofactors are present in all the Nars isolated so far with the only exception of the membrane bound Nar from *Pseudomonas nautica* 617 (*Pn* NarGHI), in which preliminary studies seem to suggest that one of the hemes *b* of the NarI subunit is replaced by a *c*-type heme (Correia et al., to be published).

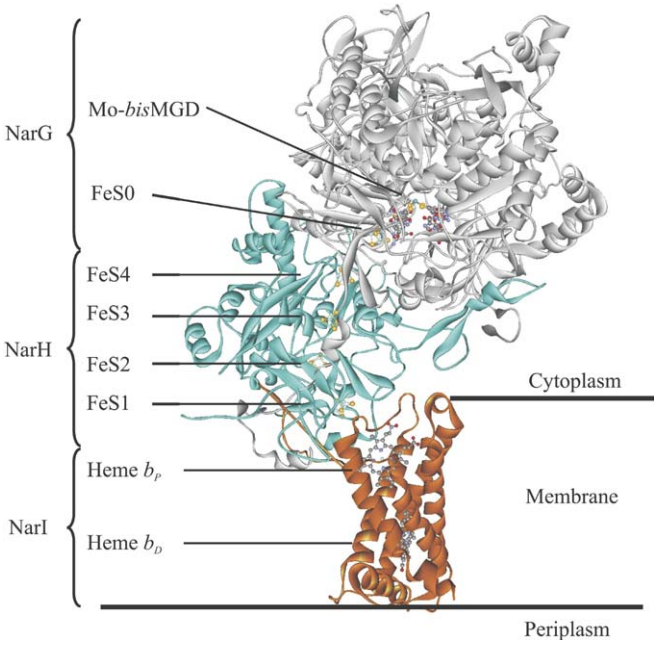

Fig. 3. Overall three-dimensional structure of NarGHI from *E. coli* K12. The names of the respective subunits together with the metal cofactors are indicated.

The structure of NarGHI reveals that the active site is composed of a molybdenum atom coordinated to four sulfur atoms from two pterin cofactors, as found in all the members of the DMSO reductase family, and bidentate coordination with the oxygen atoms from the side chain carboxylate group of an aspartate molecule (Asp222) (Fig. 2) [26], which has not been observed so far in a mononuclear Mo-enzyme. The soluble NarGH shows a similar active site structure with two pterin molecules but, in contrast, the coordination with aspartic acid is through only one oxygen with the sixth coordination position being occupied by an oxo group at 1.8 Å (Fig. 2) [25]. These differences cannot be attributed to the redox state of the sample since both NarGHI and NarGH were crystallized in aerobic conditions and therefore structures must represent oxidized forms of the enzymes. The coordination of metal sites by side chain carboxylates from aspartic and glutamic acid molecules has been the subject of several studies [16]. These studies showed that carboxylate groups present high flexibility as also suggested by Jormakka et al. [25], which is reflected in these two crystallized forms of *Ec* Nar. Another novel aspect of Nar is the unusual coordination of FeS0 by one histidine and three cysteines, which was found only once before in *Dg* hydrogenase [25,26,28].

The redox and EPR (electron paramagnetic resonance) properties of Nar from several sources have been the subject of several studies [18,20,24,29–31]. Nars show two types of pH-dependent Mo(V) ion EPR signals, which show, in addition, resonance lines split by a solvent exchangeable proton. The Mo(V) ion species associated with these signals were named the low-pH ($g_{av} = 1.984$ and $A_{av} = 9.3$ G) and the high-pH ($g_{av} = 1.977$ and $A_{av} = 3.4$ G) forms. The molecular structure of the active site of NarGHI does not show an hydroxyl/water ligand [26], as one would expect from the EPR data, and the coordination of the Mo site in NarGH presents an Oxo ligand and one oxygen from a carboxylate (Fig. 2) [25]. These results indicate that bidentate coordination is open upon reduction to Mo(V) giving rise to the solvent exchangeable low- and high-pH forms of the enzymes. Recent studies performed in *Pn* NarGHI shows, besides the low and high-pH signals, two novel EPR signals with no differences in D_2O exchanged samples that are obtained upon nitrate oxidation of reduced enzyme (Correia et al., to be published). These two species might be associated, in principle, with Mo(V) species having the molecular structure seen in both crystal structures of *Ec* Nar. The catalytic involvement of all these Mo(V) species is far from being elucidated and additional work is necessary to clarify their roles [18,29].

EPR studies in *Ec* Nar detected the presence of only four FeS centers while the structures of NarGH and NarGHI demonstrated the existence of five FeS centers. The EPR signal of the [3Fe–4S] cluster ($g_{av} = 2.00$) and one additional signal with two components (major and minor) was attributed to a single [4Fe–4S] center in different conformations (major, $g_1 = 2.049$, $g_2 = 1.947$, $g_3 = 1.870$ and minor, $g_1 = 2.010$, $g_2 = 1.885$, $g_3 = 1.871$) [30–32]. The EPR properties of the remaining EPR-detected two centers could not be determined precisely because of magnetic interaction between centers, which broaden the spectra making it de-convolution difficult. Recently, EPR studies by Rothery et al. performed in a NarG mutant that lacks the molybdenum site showed that the fifth FeS (FeS0) center is a [4Fe–4S] cluster in a high-spin configuration ($g_1 = 5.556$, $g_2 = 5.023$) [33].

2.2. Gene organization, expression control, and mechanism of action

In *Escherichia coli*, genes that code nitrate reductase are grouped in the operon *narGHJI*, which is located in the *chlC* locus of the chromosome. NarJ is a polypeptide that participates in NarGH assembly prior to its attachment to NarI in the membrane [34]. The expression of *nar* genes are nitrate/nitrite regulated by a signal transduction system of kinases-phosphatases, involving the operons *narXL* and *narQP* (Fig. 4B) [35]. The operon *narXL* is located in the *chlC* locus but *narQP* is coded out. NarX and NarQ are homologous membrane proteins that detect periplasmic nitrate and nitrite and then activate the NarL and NarP regulators by phosphorylation [36–39]. In this conformation, the regulators bind specific DNA consensus sequences called NarL heptamers [40]. The operons *narGHJI* (respiratory nitrate reductase), *frdABCD* (fumarate reductase) and *narK* are only under the control of NarL, while both NarL and NarP regulate *nrfABCDEFG* (nitrite reductase) and *napFDAGHBC* (periplasmic nitrate reductase) [37]. This is consequently, a complex regulatory system that detects the nitrate:nitrite ratio, inactivating NarL when nitrate is in low levels, and favoring nitrite consumption. There is an additional Nar in *E. coli*, coded in the *narZWYV* operon in the *chlZ* locus, which is expressed constitutively at low levels, and its proposed role is to cushion bacterium respiration when culture lacks oxygen [35].

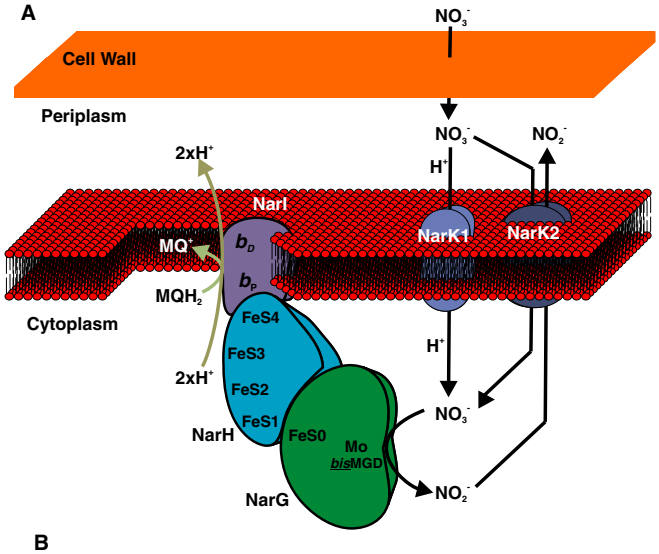

Fig. 4. (A) Respiratory nitrate reduction by Nar enzymes. (B) Gene organization of the *nar* gene clusters. Note the symbols "-" (short distance in the DNA sequence) and "-//-" (long distance in the DNA sequence).

Nars in other bacteria such as *Paracoccus denitrificans* and *Paracoccus pantotrophus* are coded by operons with the same composition (Fig. 4B). These operons code a Nar having basically the same molecular structure, but that differs in respect to those in *E. coli* in how they control the expression system, with the transcription being under the control of the FNR-like regulator NarR [41].

The proposed mechanism for the reduction of nitrate to nitrite in *E. coli* K12 is schematized in Fig. 4A. The MQH_2 situated in the outer part of the cytoplasmic membrane are oxidized by NarI liberating 2 protons into the periplasm. The two electrons are conducted through the electron transfer pathway of the NarGHI complex to the Mo-*bis*MGD site, where nitrate is reduced consuming two cytoplasmic protons. As the catalytic subunit of Nar is cytoplasmic, nitrate must be introduced into the cell. This is performed by two transporters. The proton:nitrate symporter NarK1 is activated at the beginning of the nitrate respiration and the co-transported protons are consumed in the nitrate reduction. Once the reaction accumulates nitrite, the function of NarK1 is substituted by NarK2, which acts as nitrate:nitrite antiporter, allowing the maintenance of the steady state [42]. This membrane process, when coupled to formate oxidation generates the proton motive force (PMF) necessary for ATP synthesis [6,43].

3. Periplasmic nitrate reductases

3.1. Molecular and spectroscopic properties

NapA from *D. desulfuricans* (*Dd* NapA) was the first reported structure for a periplasmic nitrate reductase (Fig. 5) [44,45]. *Dd* Nap is a monomeric protein of ellipsoidal shape with an α/β type fold organized in four domains, all involved in cofactor binding. The active site is a hexacoordinated Mo atom with four sulfur atoms from two pterin ligands, one hydroxo/oxo group and sulfur from a cysteine as ligands (Fig. 2). In addition, the protein has an iron–sulfur cluster of the type [4Fe–4S], which is supposed to be involved in electron transfer. A funnel-like cavity is formed from the surface to the catalytic site (~15 Å) due to the spatial arrangement of domains II and III. The hydroxo/oxo ligand of the molybdenum atom, which is

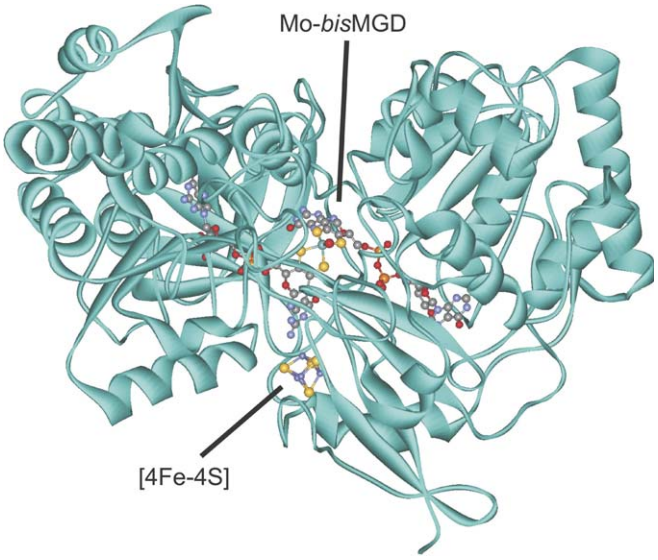

Fig. 5. Overall three-dimensional structure of NapA from *Desulfovibrio desulfuricans* ATCC 27774.

supposed to be the position where the substrate binds, points into this channel, suggesting that nitrate entrance and nitrite exit would be via this channel. In contrast, Naps obtained from other sources such as *Rhodobacter sphaeroides* (*Rs* Nap) [46], *Wautersia eutropha* (*We* Nap) [47] and *Paracoccus pantotrophus* (*Pp* Nap) [48,49] are heterodimeric proteins with a large (80–90 kDa) and small (~17 kDa) subunits. The only crystal structure available for an heterodimeric Nap belongs to *Rs* NapAB which was determined at a resolution of 3.2 Å [50,51]. The arrangement of the catalytic subunits of *Dd* NapA and *Rs* NapA are very similar in terms of metal cofactor content, global fold and domain organization, with a r.m.s.d. of 1.26 Å for the 683 Cα atoms. However, their primary sequences reveal a low identity (~35%), but the Mo-*bis*MGD catalytic site and [4Fe–4S] cluster binding residues are conserved along with the amino-acids that mediate the electron flow from the [4Fe–4S] center to the molybdenum [52,53]. The small subunit NapB, which is present in most Naps but absent in *Dd* Nap, was recently determined at a higher resolution (1.25 Å) using the recombinant NapB protein from *Haemophilus influenzae* through MAD (multiwavelength anomalous dispersion) methods [54]. A structural comparison between all these proteins was recently reported by Moura et al. [27].

EPR studies performed in *Pp* NapAB indicate a Mo-site with high coordination flexibility as shown by the different EPR active species obtained with the enzymes poised at different redox potentials and in the presence of different inhibitors [55–59]. The enzyme in the as-prepared form shows a Mo(V) EPR signal (high *g* resting) typical of Mo(V) ion species ($g_1 = 1.998$, $g_2 = 1.990$, $g_3 = 1.981$) split by two non exchangeable species with nuclear spin $I = 1/2$, presumed to be the β-methylene protons from the coordinated cysteine seen in the crystal structure of *Dd* Nap ($A_{av} = 5.5$ G, $A_{av} = 2.8$ G). A similar resting EPR signal was detected in *Rs* NapAB [50]. Under turnover conditions, the resting signal is replaced with a new Mo(V) ion signal (high *g* nitrate) showing a hyperfine split with a single non exchangeable proton ($g_1 = 1.999$, $g_2 = 1.989$, $g_3 = 1.981$, $A_{av} = 5.1$ G), which was suggested to be a catalytic intermediary. This signal is similar to that obtained in *Dd* Nap when reduced samples are re-oxidized with nitrate [60]. Recent results on DdNapA also show similar EPR signal in some cases in the presence of inhibitors (González et al., to be published). These preliminary data obtained in both *Dd* Nap and *Rs* Nap suggests that the active sites of the three enzymes are similar. Nevertheless, this is in contradiction with the coordination sphere deduced from EXAFS studies performed in *Pp* Nap, which indicates that besides the bi-pterin coordination, the active site presents three extra ligands. Additional work should be performed to clarify these discrepancies.

3.2. Genes organization, expression control, and mechanism of action

The *nap* genes have been identified in several prokaryotic organisms (Fig. 6B) [15,46,47,61–64]. In contrast with *nar* operon, the *nap* operons present heterogeneity in gene composition as well as in ordering. Nine different genes have been identified as components for operons that code Naps in different organisms.

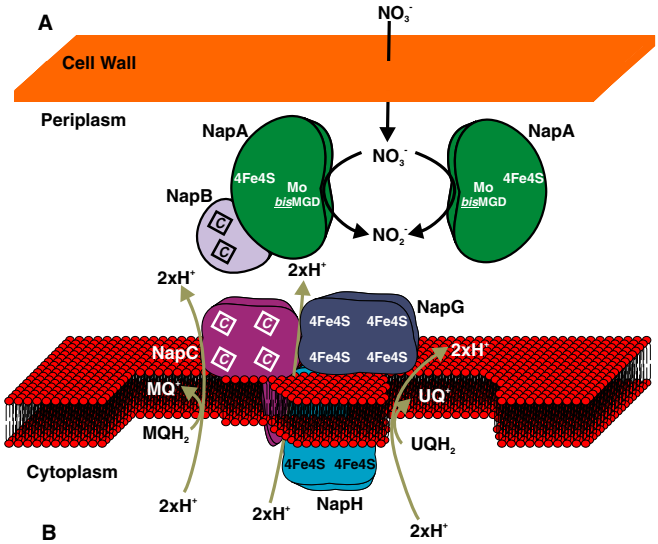

Fig. 6. (A) Dissimilatory nitrate reduction by Nap enzymes. (B) Gene organization of the *nap* gene clusters.

The *napA* gene codes the catalytic subunit NapA that contains the Mo-*bis*MGD active site and a FeS center. The assembling of the apo-protein with the metal cofactors is carried out in the cytoplasm. Then, the folded holo-protein is transported to periplasm by the TAT (Twin Arginine Translocator) system by recognizing the signal-peptide present in NapA [65]. However, this is not valid for all the catalytic subunits since although NapA from *Pseudomonas* G-179 is found in the periplasm, it lacks this signal peptide. Once in the periplasm, NapA and the product of *napB* gene are assembled to give the heterodimeric NapAB. NapB is secreted into periplasm by the general secretory pathway [66]. This process occurs for all the Naps except for *Dd* Nap, which is obtained in monomeric form [60]. This should also be the case for *D. hafniense* DCB-2 Nap, whose *nap* operon and whole shotgun genomic sequence does not show the presence of the *napB* gene.

Almost all the *nap* operons code the protein NapC (Fig. 5B), which is a *c*-type tetra-haem membrane-anchored protein (~25 kDa) belonging to the NapC/NirT family [67–69]. This protein is involved in the electron transfer from the quinol pool to periplasmic reductases. When NapC is not coded in the *nap* operon, its function is substituted by another protein of the NapC/NirT family which is coded out of the operon and is expressed under denitrifying conditions. Furthermore, some operons code the ferredoxins NapG and NapH. NapG is a periplasmic protein having $4 \times$ [4Fe–4S] clusters. NapH is an integral membrane protein with $2 \times$ [4Fe–4S] exposed to the cytoplasm [70,71].

The remaining genes, *napD*, *napE*, *napF*, *napK* and *napL* code for different proteins that are not directly involved in the nitrate reduction. NapD is a cytoplasmic protein that belongs to the TorD family, which act as chaperones and are always present in operons that encodes molybdoenzymes [72]. NapF is a cytoplasmic iron–sulfur containing protein with four loosely bound [4Fe–4S] clusters, which is thought to participate in the assembling of the iron–sulfur cluster of NapA [70,73]. The other genes code for proteins with so far unknown functions.

The high diversity of *nap* operons suggest that nitrate reduction by Naps is not unique as in the case of Nars. Two proposed electron transfer pathways involved in nitrate reduction by periplasmic nitrate reductases are depicted in Fig. 6A. One proposed for the Nap complex from *E. coli* K12, is used by the cell to support anaerobic metabolism as an alternative to the Nar pathway when nitrate concentration is low in the culture medium [74–79]. This pathway is used for nitrate respiration in bacteria that lack Nar such as *D. desulfuricans* [60], *Pseudomonas* sp. G-179 [64] and *B. japonicum* [61,80]. As shown in Fig. 6A(right), the electrons necessary for nitrate reduction are obtained from the UQH_2 pool ($E^0 = +90$ mV), and then transferred to NapAB (NapA in enzymes lacking NapB) through an electron transfer chain mediated by the metal centers of NapHGC. This process implies the oxidation of two UQH_2 molecules resulting in the translocation of $2H^+$ to the periplasm. The transport of two electrons from NapG to NapC would also be accompanied with translocation of two additional protons [71], which gives a net balance of four translocated protons, of which two are consumed in the nitrate reduction. The energetic balance for each nitrate molecule converted through the Nap complex would be as efficient as the Nar pathway since both systems produce a net gradient of two protons [6,81]. This proposed mechanism should also occur in *Haemophilus influenzae* and *Shewanella oneidensis* MR-1 since they have the necessary genes to accomplish this function.

The second pathway is operative in organisms that express NapC but lack genes *napG* and *napH* (*P. pantotrophus*, *W. eutropha*, *R. sphaeroides* and *R. capsulatus*). The electron transfer pathway between NapAB and the QH_2 pool is similar to the previous pathway, but is mediated only by NapC. The electrons necessary for nitrate reduction are provided by the MQH_2 pool (Fig. 6A Left), since NapC is unable to use UQH_2 [71]. As the two protons translocated to the periplasm in the MQH_2 oxidation are consumed in the nitrate reduction, this process gives no net proton gradient. Therefore, nitrate reduction is used in these organisms as an electron sink to eliminate an excess of reducing equivalents [47,82–87] accumulated in the cytoplasm as NADH and $FADH_2$.

The *nap* operons of other bacteria that lack the necessary genes to perform nitrate reduction by any of these two pathways must use an alternative way or a modified mechanism of those proposed above. For instance, the *nap* clusters from *B. japonicum* and *Pseudomonas* sp. G-179 lack the *napGH* genes but are maximally expressed under denitrifying conditions [61,64]. Another example is the *nap* cluster from *W. succinogenes* that lacks NapC but codes the *nrfH* and *fccC* genes in its genome which codes for proteins of the NapC/NirT family. The *knockout* of these genes does not abolish nitrate reduction through the Nap complex; suggesting that this bacterium uses an alternative pathway to transfer the electrons to NapAB [88]. The *nap* cluster from *D. hafniense* DCB-2 lacks both NapB and NapC. However, a protein of the NapC/NirT family but not NapB can be found in the genome sequence. This would indicate that, as in *Dd* Nap, the periplasmic protein is a monomer that can receive electrons either directly from the tetrahemic membrane-anchored protein or through a periplasmic soluble mediator such as a cytochrome or a flavin. Clearly, the mechanisms involved in the periplasmic nitrate reduction are not general and require additional investigation.

4. Assimilatory nitrate reductases

4.1. Molecular and spectroscopic properties

Nas is the first enzyme involved in the anabolic pathway of nitrogen assimilation (Fig. 1). These enzymes along with the *nas* genes have been reported from several bacteria. All the Nas isolated so far have an active site containing a

Mo-*bis*MGD cofactor but the molecular properties and the number and type of electron transfer centers are diverse and vary in the different organisms. Nas from *Klebsiella pneumoniae* and *Rhodobacter capsulatus* are heterodimers made up of a large catalytic subunit (90–105 kDa) and a small subunit (~45 kDa). The catalytic subunit contains, besides the active site, two FeS centers; one [4Fe–4S] and one [2Fe–2S], whereas the small subunit contains a FAD cofactor that uses NAD(P)H as an electron donor. In contrast, the catalytic subunit of *Bacillus subtilis* Nas contains the Mo-*bis*MGD cofactor and only one [4Fe–4S] center, whereas the small subunit holds $2\times$[2Fe–2S] centers and one FAD cofactor. Monomeric Nas has been found in *Azotobacter vinelandii* and *Cyanobacteria*. These proteins have molecular weights in the range 75–105 kDa and contain a Mo-*bis*MGD cofactor and one [4Fe–4S] center. The physiological electron donor is flavodoxin in *Azotobacter vinelandii* and flavodoxin and ferredoxin in *Cyanobacteria* [5,8].

At present, no crystal structure has been reported for any assimilatory nitrate reductase. Monomeric Nas from cyanobacteria is closely related to *Dd* Nap, which is in line with phylogeny calculations that indicate that Nas proteins are more related to Naps than Nars [17].

NarB from the cyanobacteria *Synechococcus* sp. Strain PCC 7942 represents the best spectroscopic characterized assimilatory nitrate reductase so far. Note that the notation Nar is used to identify an assimilatory nitrate reductase. EPR studies confirmed the presence of one [4Fe–4S] cluster/molecule having a midpoint redox potential of −190 mV (vs. normal hydrogen electrode, NHE) for the $[4Fe–4S]^{2+/1+}$ couple. EPR characterization of the Mo center revealed similar EPR signals to those obtained in periplasmic nitrate reductases but obtained with the samples poised at different redox potentials and conditions. Dithionite reduction of NarB yields a Mo(V) signal similar to the High *g* [resting] reported for *Pp* Nap whereas oxidation with air of this sample shows an EPR signal similar to that obtained by treating *Pp* Nap with cyanide, indicating that despite both enzymes yielding similar Mo species, they have different redox properties. EPR redox titration of the "High *g*" Mo(V) species reveal midpoint redox potentials of −150 and <−550 mV for the $Mo^{6+/5+}$ and for $Mo^{5+/4+}$ couples, respectively. These values suggest that the Mo center is reduced to Mo(IV) only after the nitrate binds to Mo(V), which raises the midpoint redox potential of the $Mo^{5+/4+}$ couple to −350 mV [89]. This increase in the $Mo^{5+/4+}$ redox potential triggers the electron transfer from the reduced iron–sulfur cluster to the molybdenum, providing the two electrons required for nitrate reduction to nitrite.

4.2. Genes organization, expression control, and mechanism of action

The *nas* genes are clustered in operons with different compositions as observed in Naps (Fig. 7B). The expression of this operon in both *K. pneumoniae* and *R. capsulatus* was found to be insensitive to oxygen but induced by nitrate and nitrite, and repressed by the presence of ammonium in the culture medium. For example, in both *K. pneumoniae* and *R. capsulatus*, the *nasR* gene located upstream from the *nasFEDCBA* operon encodes a transcription antiterminator, which is a positive regulator subject to the presence of nitrate and nitrite. The *nasFEDCBA* operon encodes a typical ABC-type nitrate/nitrite transporter in *nasFED*, a siroheme assimilatory nitrite reductase in *nasB*, and the NADH-dependent assimilatory nitrate reductase, where *nasC* encodes the small FAD-containing subunit and *nasA* the catalytic subunit (Fig. 7A). In contrast, the catalogue of genes in *B. subtilis* is different (Fig. 7B). The *nasBC* genes code the small and the catalytic subunits, respectively, a nitrate transporter is encoded in *nasA*, and a soluble NADH-nitrite reductase is expressed from the *nasDE* genes. Furthermore, this cluster has *nasF*, a gene involved in the biosynthesis of heme cofactors. A different situation is found in the *genus Cyanobacteria*, which, as seen above, produces monomeric nitrate reductase NarB. Usually, *narB* gene is co-transcribed with the *nrtABCD* nitrate transport genes and the nitrite reductase *nirA*.

Because of the cytoplasmic localization of the enzyme, nitrate reduction takes place strictly in the cytoplasm and it therefore has to be transported into the cell. The nitrate uptake is performed by specific transporters, which are usually coded, as seen above, in the same genomic region of *nas* genes. Once in the cytoplasm, the nitrate anion is reduced at the catalytic site in Nas releasing nitrite; which

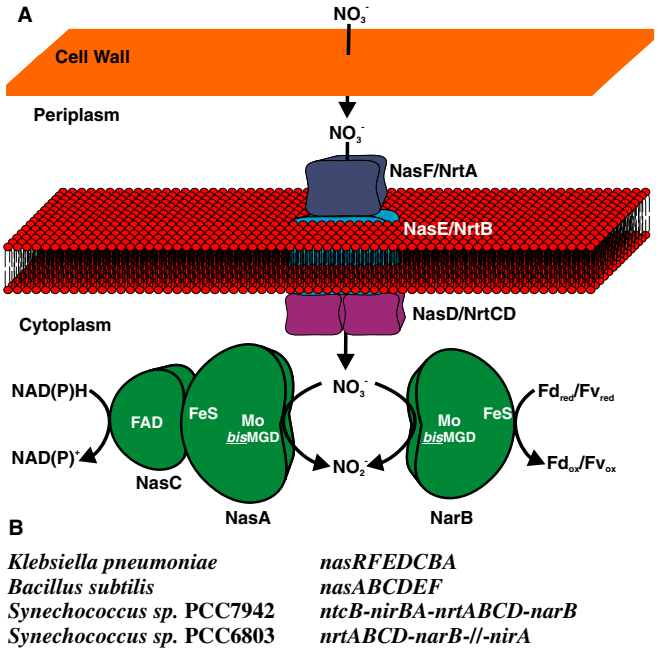

Fig. 7. (A) Pathway for nitrate assimilation by Nas enzymes. The figure depicts the enzymes of the *Klebsiella*/*Rhodobacter* (left) and *Cyanobacteria* (right) groups. (B) Organization of the gene clusters from the discussed examples. The meaning of the symbols "-" and "-//-" are given in the caption of Fig. 4.

is exported to the periplasm and immediately reduced to ammonium in a six-electron reaction catalyzed by Nir. This ammonium is then transferred to the cytoplasm and incorporated into biomolecules through the Glutamine Synthetase and GOGAT pathways (Fig. 1) [5,8].

5. Summary and outlook

Nitrate reduction is performed in prokaryotic organism by three different enzymes, which can be differentiated via their different functions, localization in the bacterium cell, and molecular properties. Based on the molecular structure of the active site in *Dd* Nap, the proposed reaction mechanism for nitrate reduction implies the replacement of a OH/H_2O ligand by a nitrate molecule (see Fig. 2), which is then reduced to nitrite. In this process, the electrons necessary for nitrate reduction are obtained from the Mo site which receives them from an external electron donor by means of an electron transfer reaction mediated by metal cofactors such as FeS and heme. Although the redox cofactors trace a path for electron transfer in all the structures determined so far, the redox potentials evaluated for the FeS clusters, for instance in *Ec* Nar, do not favor electron transfer. However, it is proposed that the favorable electron transfer processes override the one unfavorable step. Furthermore, EPR and potentiometric studies in nitrate reductases reveal Mo active sites with different redox and electronic properties, despite these enzymes catalyzing a unique reaction. All this evidence suggest that some that additional conformational changes should occur with the protein during catalysis. Therefore, the evaluation of the molecular properties of these enzymes under turnover conditions could help to clarify all these still unveiled aspects regarding the mechanism of action of nitrate reductases and mononuclear Mo-containing enzymes in general.

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