
REVIEW

Nitrate Reductases: Structure, Functions, and Effect of Stress Factors

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Abstract—Structural and functional peculiarities of four types of nitrate reductases are considered: assimilatory nitrate reductase of eukaryotes, as well as cytoplasmic assimilatory, membrane-bound respiratory, and periplasmic dissimilatory bacterial nitrate reductases. Arguments are presented showing that eukaryotic organisms are capable of nitrate dissimilation. Data concerning new classes of extremophil nitrate reductases, whose active center does not contain molybdocofactor, are summarized.

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Nitrogen is a constituent of main macromolecules—proteins and nucleic acids—and is one of most vitally important elements on Earth. It is incorporated into compounds in different degrees of oxidation (from N^{5+} to N^{3-}), the interconversions of which define the global biochemical cycle of nitrogen. Inorganic nitrogen is converted to the biologically useful form during nitrogen fixation and assimilation that provide for nitrogen incorporation into the carbon backbone of organic compounds. Processes of nitrification (oxidative conversion of ammonium to nitrate) and denitrification (successive nitrate reduction to nitrite, nitrogen oxides (NO and N_2O)) are involved in nitrogen removal from the environment (Fig. 1).

Nitrate reduction, the most important stage of nitrogen turnover in nature, has several functions: i) utilization of NO_3^- as a source of nitrogen (nitrate assimilation); ii) production of metabolic energy during NO_3^- utilization as terminal acceptor of electrons (nitrate respiration), and iii) dissipation of excess of reducing energy to maintain oxidation—reduction balance (nitrate dissimilation).

The first, most important stage in the chain of nitrate reduction to nitrites is catalyzed by nitrate reductase

(NRase). There are four types of NRases—eukaryotic assimilatory NRase (Euk-NR) [1, 2] and three different bacterial enzymes, i.e. cytoplasmic assimilatory (Nas), membrane-bound respiratory (Nar), and periplasmic dissimilatory (Nap) NRases [3–6] (Fig. 2).

As a rule, all studied NRases share a common property—the presence of molybdenum (Mo) and molybdenum cofactor (Mo-co) in the enzyme active center. Bacterial NRases contain molybdopterin-guanine dinucleotide (MGD), whereas mononucleotide is present in the active center of eukaryotic NRases.

In bacterial MGD-binding subunit, molybdenum can be coordinated by four thiolate ligands located in two MGD halves (Fig. 3a). Molybdenum is additionally coordinated by $-S$, $-O$, or $-Se$ bonds of cysteine, serine, or selenocysteine residues of the polypeptide chain, as well as by accessible oxo ($-O$) and hydroxo ($-OH$) groups or by water (Fig. 3b) in different molybdenum enzymes [7–10].

Reactions catalyzed by dimethylsulfoxide (DMSO) and trimethylamine N-oxide (TMAO) reductases follow the oxo-transferase mechanism, in which oxo-group on the oxidized ion Mo(VI) loses OH^-/H_2O upon reduction to Mo(IV). Nitrate is able to bind Mo in the reduced state and undergo reduction to nitrite, which regenerates the oxo-group on release of Mo(VI) (Fig. 4).

Structures of some catalytic subunits of such MGD family members as formate reductase [8], DMSO reductase [10], TMAO reductase [9], and Nap from *D. desulfuromonas* [7] have been revealed. However, the number of Mo-bound oxo-groups still attracts attention of

Abbreviations: DMSO) dimethyl sulfoxide; Euk-NR) eukaryotic assimilatory NRase; MGD) molybdopterin-guanine dinucleotide; Mo) molybdenum; Mo-co) molybdenum cofactor; Nap) periplasmic dissimilatory NRase; Nar) membrane-bound respiratory NRase; Nas) cytoplasmic assimilatory NRase; NRase) nitrate reductase; TMAO) trimethylamine N-oxide.

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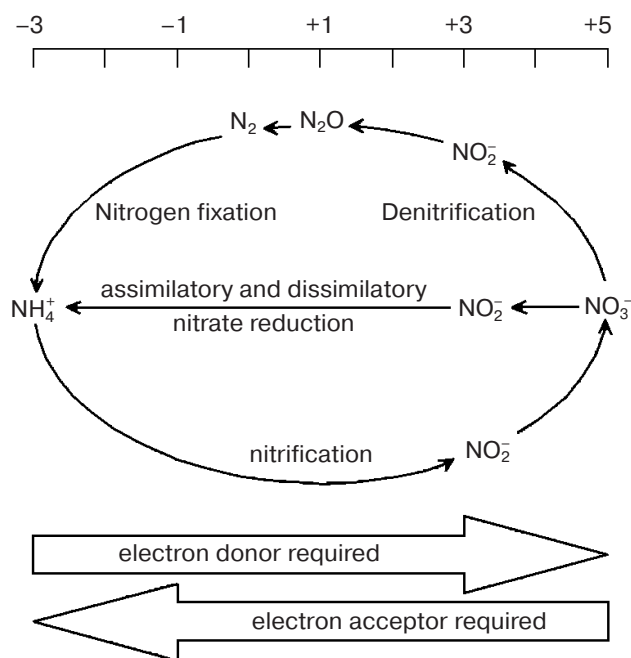
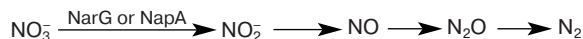
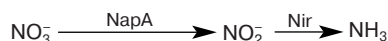
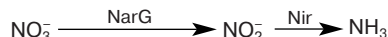


Fig. 1. Biogeochemical cycle of nitrogen turnover.

Denitrification

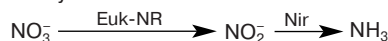


Dissimilatory reduction of nitrate to ammonium



Assimilatory reduction of nitrate to ammonium

Eucaryotes



Prokaryotes

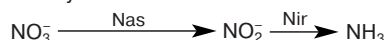


Fig. 2. Reactions of nitrogen reduction in pro- and eukaryotes.

researchers. Studying periplasmic NRase (Nap) in *P. pantotrophus* by EXAFS (Extended X-ray Absorption Fine Structure) pointed to the existence of di-oxo-condition in Mo(VI) [11], whereas the Nap crystalline structure in *D. desulfuricans* suggests mono-oxo condition in Mo(VI). It should be noted that the amino acid sequence of Nap from *D. desulfuricans* is similar to that of assimilatory enzymes. There are a number of serine residues that increase the probability of Mo–O–Ser ligands in Nar compared with formation of Mo–S–Cys ligands found for Nap [7] and predicted for Nas. Structural and molecular-genetic peculiarities of different type NRases will be considered below.

ASSIMILATORY BACTERIAL NITRATE REDUCTASE (Nas)

Two classes of assimilatory NRases have been found in bacteria: ferredoxin- or flavodoxin-dependent Nas and NADH-dependent Nas, the classification of which is based on the cofactor structure (Fig. 5).

Ferredoxin- or flavodoxin-dependent bacterial and cyanobacterial NRases contain in their active center Mo-co and [Fe-S] clusters and are devoid of FAD or cytochromes [12, 13]. NRases from *Azotobacter vinelandii* and *Plectonema boryanum* consist of a single 105 kD subunit incorporating a [4Fe-4S] center and molybdenum (1 atom/molecule) and use flavodoxin as a physiological donor of electrons [14]. Analysis of amino acid sequence of these enzymes has shown the presence of cysteine residues at the N-terminus, which evidently binds the [4Fe-4S] center. Ferredoxin-dependent NRases have been found in *A. chroococcum*, *Clostridium perfringens*, and *Ectothiorhodospira shaposhnikovii* [1].

NADH-dependent NRases of *Klebsiella oxytoca* (*pneumoniae*) and *Rhodobacter capsulatus* are heterodimers consisting of the diaphorase FAD-containing subunit of 45 kD and a catalytic subunit of 95 kD with molybdenum cofactor and N-terminal [4Fe-4S] center [15]. Besides, NRase of *Klebsiella oxytoca* (*pneumoniae*) contains an additional [2Fe-2S] center bound to a C-terminal cluster of cysteine residues, which resembles by its amino acid sequence the NifU protein, the *nifU* gene product, involved in formation of [Fe-S] clusters in the nitrogenase active center [16].

This region is absent from the ferredoxin-dependent NRase, whereas in the NADH-dependent NRases it may play the role of the ferredoxin-similar domain carrying out electron transfer. The NADH-dependent NRase of *Bacillus subtilis* does not contain the NifU-like domain in the catalytic subunit, but contains two tandem NifU-like regions in the diaphorase subunit, which may be involved in [4Fe-4S] or [3Fe-4S] binding [17].

Regulatory and structural genes responsible for nitrate and nitrite uptake (transfer) and reduction are usually located in the same operon. The *nasFEDCBA* operon of *K. oxytoca* M5al encodes proteins responsible for nitrate and nitrite assimilation [18, 19], and in this case genes *nasFED* encode a periplasmic carrier that provides for nitrate and nitrite transfer [19], the NADH-dependent NRase is encoded by genes *nasC* (diaphorase subunit) and *nasA* (catalytic subunit), whereas gene *nasB* is responsible for assimilatory nitrite reductase [20].

The operon encoding enzymes responsible for nitrate assimilation is also well-studied and characterized in *A. vinelandii*. Structural genes encoding flavodoxin-dependent NRase of *A. vinelandii* (*nasB*) are transcribed together with gene *nasA* responsible for synthesis of nitrite reductase [20]. In most other studied bacteria, genes *nirA*, *nrtABCD*, and *narB*, responsible, respectively, for NRase

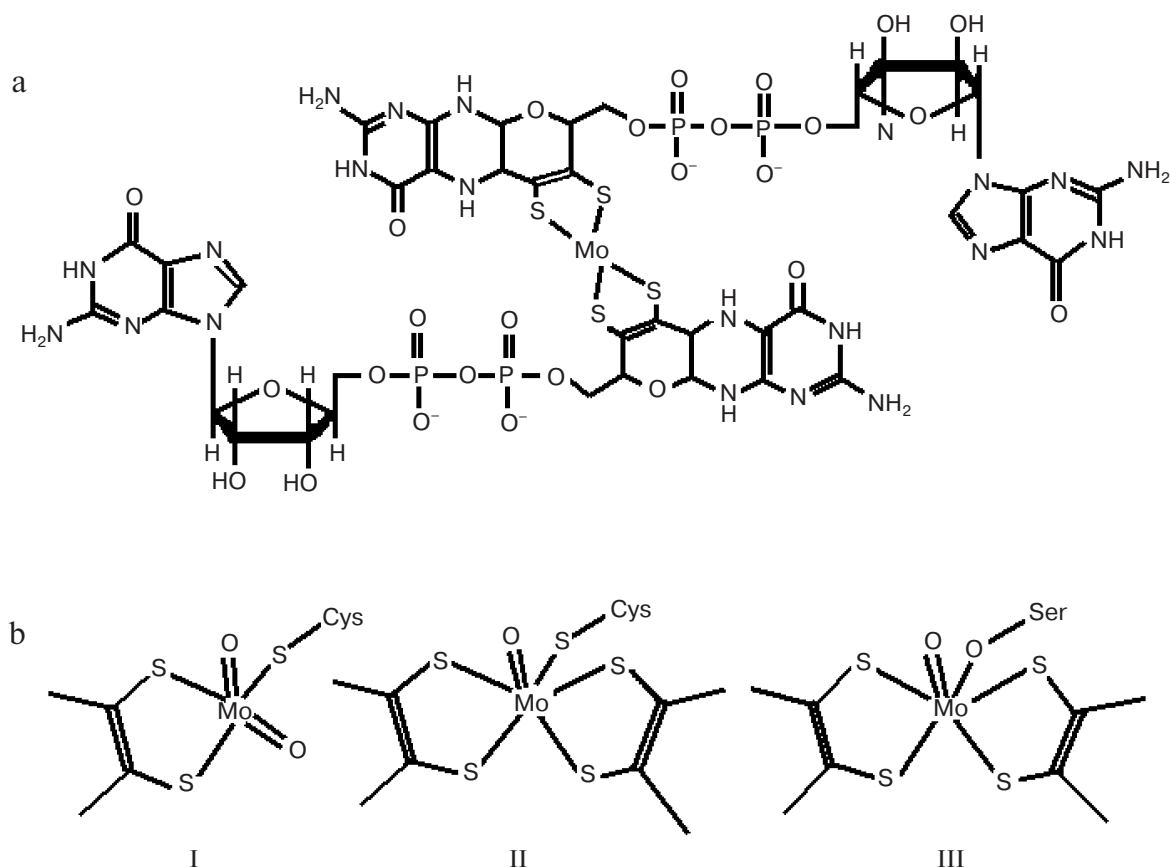


Fig. 3. Structure of molybdopterin-guanine-dinucleotide cofactor (MGD cofactor) (a) and active center of NRase (b): I, eukaryotic (Euk-Nar); II, periplasmic (Nap); III, membrane-bound (Nar).

synthesis, nitrate transport, and nitrite reductase synthesis, make up a single operon [21, 22].

Nitrate transport into a cell involves carriers of the NarK family and ATP-dependent transporters of the ABC family (ATP-binding cassette transporters) [18, 23].

Bacillus subtilis is able to grow using nitrate as a unique source of nitrogen. Assimilatory nitrate and nitrite reductases are encoded by the locus containing genes *nasABCDEF*. The *nasA* gene mutant was not able to grow

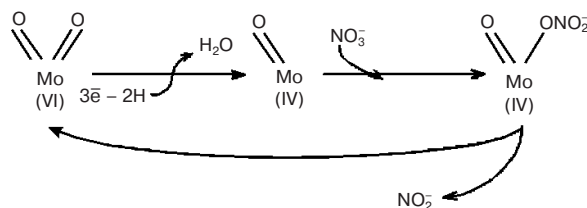


Fig. 4. Scheme of catalytic cycle of nitrate reduction by Nap of *P. denitrificans*.

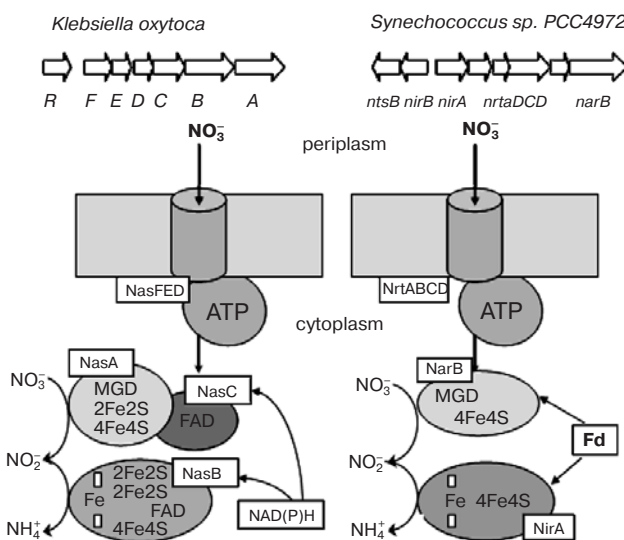


Fig. 5. Organization of genes encoding nitrate assimilation in bacteria *Klebsiella oxytoca* and *Synechococcus* PCC7942.

Another membrane-bound NRase was found in *E. coli* (and in this organism only) [30]. It was shown by denaturing electrophoresis in polyacrylamide gel that NarZYV consists of two subunits with molecular mass of 150 and 60 kD encoded by *narZ* and *narY* genes and contains Mo-co. Subunit γ of NarZYV encoded by *narV* was lost during purification, but its existence was proved by spectral methods. The [Fe-S] clusters of NarZYV resemble by their characteristics NarGHI, except the potential of the α -subunit high-potential centers. Both enzymes catalyze nitrate and chlorate reduction and are inhibited by azide.

Despite common properties, NarGHI and NarZYV are characterized by different electrophoretic mobility ($R_f = 0.23$ and 0.38 for NarGHI and NarZYV, respectively). NarZYV is present in *E. coli* cells in minimal amounts independently of the oxygen partial pressure.

Nitrate reduction is known to be controlled by oxygen at several levels: at the level of gene expression and at the level of nitrogen oxo-anion transport. In *E. coli*, *P. fluorescens*, and *P. stutzeri* the *narGHJI* operon expression is induced by anaerobiosis and by the presence of nitrate or nitrite [40–42]. The regulatory transcription factor Fnr (regulator of fumarate- and NRases) isolated from *E. coli* is an anaerobic activator of *narGHJI* and in structure is a dimer containing [Fe-S] clusters. The protein is inactivated by molecular oxygen as a result of disintegration of [4Fe-4S] clusters and is converted to the monomer form. The Fnr protein monomer can be divided into three functional domains: N-terminal sensor region incorporating the [4Fe-4S] cluster (residues 1–50), central allosteric domain contacting RNA polymerase, and C-terminal regulatory domain incorporating the H–T–H region [43]. In *P. aeruginosa* and some other *Pseudomonas* species the Anr protein (anaerobic regulation of anaerobic deiminase and NRase) was identified as a structural and functional homolog of Fnr [44, 45]. This regulator is necessary for denitrification cascade induction during growth on nitrogen oxides [44–46].

Despite significant interest of researchers in the mechanism of nitrate uptake by denitrifying bacteria, this problem is still poorly studied. In a number of articles different mechanisms of nitrate penetration were proposed such as passive uniport, ATP-dependent uniport, transmembrane potential-dependent NO_3^-/H^+ symport, and $\text{NO}_3^-/\text{NO}_2^-$ antiport [47].

BACTERIAL DISSIMILATORY PERIPLASMIC NITRATE REDUCTASE (Nap)

It is supposed that nitrate reduction in the periplasm plays the key role in aerobic denitrification in *Pseudomonas* sp. [48], *Paracoccus pantotrophus* [49, 50], and *Rb. sphaeroides* f. sp. *denitrificans* [51, 52], in adaptation to growth under anaerobic conditions in *Ralstonia eutropha* [53], in the maintenance of the oxidation–

reduction equilibrium upon nitrate usage as electron acceptor for reducing energy removal in *Rb. capsulatus*, *Rb. sphaeroides*, and *Thiosphaera pantotropha* [54, 55], or for doubling the nitrate trace amounts in the case of *E. coli* growing on a nitrate-deficient medium [56–58]. The *nap* genes were also identified in *Ps. putida* [59], *Ps. aeruginosa* [60], *Ps. stutzeri*, *Shewanella putrefaciens*, *H. influenzae*, and quite recently in some pathogenic bacteria like *Salmonella typhimurium*, *Vibrio cholerae*, *Yersinia pestis*, and *Campylobacter jejuni*. Evidently, as soon as new information concerning genomes of still not studied bacteria will become available, we can expect a significant increase in the number of species incorporating *nap* genes.

NRases localized in the periplasm are heterodimers consisting of a catalytic 90-kD subunit, incorporating molybdenum cofactor and one [4Fe-4S] center, and of a 13–19-kD subunit incorporating two-heme cytochrome *c* [61–63] (Fig. 7). They are not involved in formation of transmembrane potential.

Periplasmic NRase (NapABC enzyme) is encoded by the *napFDAGHBC* operon, in which *napA* is responsible for synthesis of the catalytic subunit, and *napB* is the two-heme cytochrome *c* involved in electron transport onto molybdopterin [64]. Both subunits are synthesized

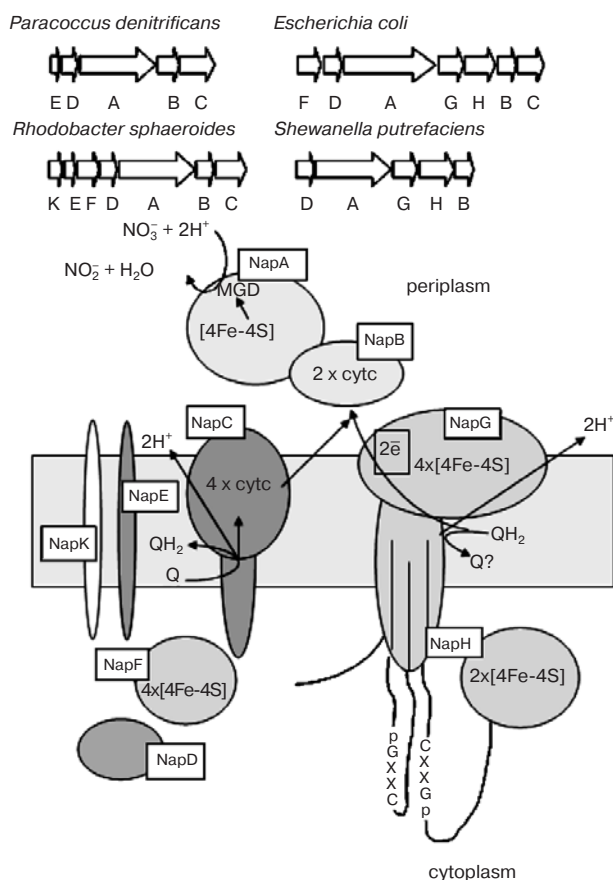


Fig. 7. Organization of genes encoding proteins of periplasmic bacterial nitrate reductase.

as a precursor with N-terminal signal sequence that defines their transfer through cytoplasmic membrane into the periplasmic space. The NapC, membrane-bound tetra-heme protein of cytochrome *c*-type, belongs to the NapC/NirT family of periplasmic multi-heme proteins of cytochrome *c*-type, which provide for the main, cytochrome *bc*₁-independent pathway of electron transfer between quinone of cytoplasmic membrane and water-soluble periplasmic oxidoreductases. The NapC/NirT protein, a homolog of NrfH protein, which transfers electrons between quinone and cytochrome *c*-nitrite reductase, was identified in *Wolinella succinogenes* [65]. The cytoplasmic protein NapD is important for NapA maturation [57]. Five different *nap* genes in different combinations with *napDABC* were detected in well-studied *nap* operons. They include *napF*, *napG*, and *napH* encoding the [Fe-S] oxidation–reduction proteins, *napE* encoding an integral membrane protein whose function is unknown, and *napK* found only in *Rb. sphaeroides* [51].

Bacterial periplasmic NRase exhibits various physiological functions and is expressed under different conditions, although usually the NRase activity is revealed under aerobic conditions, and the enzyme synthesis is not repressed by ammonium [66, 67]. In *E. coli* maximal expression of NRase is observed at low nitrate concentrations under anaerobic conditions, in this case both Fnr and NarP proteins are required [68–70]. On the other hand, the Nap system of *Ralstonia eutropha* is maximally expressed under aerobic conditions in the stationary growth stage and is not induced by nitrate [53]. In *P. pantotrophus* maximal *nap* expression was found in cells grown under aerobic conditions on such reduced carbon sources as butyrate or caproate, but no expression was detected in the case of growth on more oxidized substrates like succinate or malate [71]. In the bacterium *R. sphaeroides* DSM158 the Nap activity was stimulated, the enzyme synthesis took place both under aerobic and anaerobic conditions, but in the latter case it was more efficient [52, 68].

The periplasmic localization of the enzyme raises important questions concerning NRase transport. The cytochrome *c*-containing enzyme subunit (NapB) incorporates a typical N-terminal signal sequence that is necessary for Sec (secretion)-dependent transport [72, 73]. However, the catalytic NapA subunit can be assembled in the cytoplasm and exported into the periplasm in a folded conformation via a Sec-independent pathway [72]. It is supposed [73–75] that alternative transport systems may involve unusually long and containing double arginine site N-terminal signal sequences of NapA (and other periplasmic molybdoenzymes), like it was supposed for the pdf (proton driving force)-dependent pathways of thylakoid import. The MGD cofactor is known to be synthesized in the cytoplasm with involvement of five different loci (*moa*, *mob*, *mod*, *moe*, and *mog*), but there are still no data concerning the cofactor export in bacteria.

Besides, the size of cofactor and its complete internal environment in the protein [7] suggest that the MGD cofactor may be assembled in the cytoplasm before its transport. It has been shown that the cofactor incorporation into apoprotein is a prerequisite for the trimethylamine-N-oxidoreductase transfer in *E. coli* via Sec-independent pathways [76]. Recently genes have been identified in *E. coli* that are necessary for Sec-independent export of periplasmic protein that contains the MGD cofactor [77, 78].

ASSIMILATORY EUKARYOTIC NITRATE REDUCTASE (Euk-NRase)

The assimilatory NAD(P)H:nitrate reductase belongs to the NRase family of the class of molybdenum-containing enzymes, which are widespread in plants, fungi, yeasts, and algae [2, 79–81]. It is a water-soluble enzyme consisting of a polypeptide with molecular mass of approximately 100 kD, bound to the molybdenum-molybdopterin molecule (Mo-MPT), heme iron, and FAD. It is known that dimerization of the enzyme is necessary for its activity, and thus the native enzyme is a homodimer having a tendency to further dimerization to a homotetramer (dimer of dimer). The enzyme has two active centers carrying out the internal electron transfer from FAD onto the heme iron and then to Mo-MPT. On the first active center electrons are transferred from FAD via NADH (or NADPH) onto the enzyme; in the second active center two electrons are transferred from reduced Mo(IV) to nitrate and reduce the latter to nitrite and hydroxide. Eukaryotic NRase exhibits its activity with such electron acceptors as ferricyanide and cytochrome *c* by obtaining electrons directly from FAD and heme iron, respectively. The enzyme has only a limited similarity to prokaryotic NRase that also contains Mo and pterin cofactor. Bacterial cofactor (MGD) differs from eukaryotic MPT by an additional nucleotide. In bacteria, the Mo atom coordinates two pterins. All bacterial NRases contain the [4Fe-4S] oxidation–reduction centers as electron carriers from the electron donor to the enzyme. However, there is still no known eukaryotic NRase that contains [Fe-S] centers as components of the electron transfer chain.

Studying genes encoding NRase of the yeast methylotroph *Hansenula polymorpha* has shown that they are located in the same locus with genes *Yna1* and *Yna2* responsible for transcription [82]. A similar gene structure was also observed in different organisms, such as the fungus *Aspergillus* [83, 84] or algae *Chlamydomonas reinhardtii* [85, 86], but not in the fungus *Neurospora crassa* [87].

Despite the fact that many aspects of nitrate transport (NT) in eukaryotes are still not clear, it is assumed that a nitrate carrier really exists. Nitrate transport is carried out in symport with protons, the driving force of which is the transmembrane potential generated at the

expense of ATP hydrolysis by H^+ -ATPase [88]. Individual components of the NT system are regulated in a complex way including induction by nitrate and repression following a feedback mechanism (regulation by nitrate reduction products) [89, 90].

On the basis of expression condition and affinity to nitrate all carriers can be classified as constitutive or inducible with a high or low affinity to the substrate. Studying primary amino acid sequence of nitrate carriers NRT1 and NRT2 in algae and fungi has shown that they belong to the superfamily of peptide transporters (MFS, Major Facilitator Superfamily) [91]. NRT1 belongs to the subgroup of nitrate/nitrite transporters, but effectively transporting nitrate, histidine, and nitrite [92], whereas NRT2 belongs to the subgroup of proton-dependent oligopeptide transporters, transporting peptides, amino acids, nitrate, chlorate, and nitrite [88].

Recently it has been found that eukaryotic cells are able to carry out denitrification [93]. In particular, some species of yeasts and fungi are able to perform the dissimilatory reduction of nitrate to N_2O [94-96]. The fungus *Cylindrocarpum tonkinense* is able to denitrify NO_3^- despite the presence of ammonium ions in the medium, the presence of sugars being the key condition for switching on denitrification. In this case there is significantly increased level of enzymes involved in NO_3^- metabolism such as assimilatory NADPH- (or NADH)-dependent NO_3^- reductase, dissimilatory nitrite reductase, and NO reductase, but not ubiquinone-dependent dissimilatory NRase [96]. Thus, the existence in *C. tonkinense* of a new type of denitrification was shown, including assimilatory NRase at the first step.

Zhou et al. have shown that the fungus *Fusarium oxysporum* MT-811 was able to reduce nitrate to ammonium under anaerobic conditions [97]. This process was able to maintain growth of the fungus even under completely anaerobic conditions [97]. Cells grown under microaerophilic conditions contained a noticeable number of intact mitochondria [98], which agrees well with data on mitochondrial localization of the dissimilatory denitrification process [99]. It was assumed previously that soil fungi were obligate aerobes, but new data allow one to consider them as facultative aerobes (or facultative anaerobes).

Kurakov et al. have shown that dissimilatory NRase is synthesized *de novo* under anaerobic conditions in the fungus *F. oxysporum* 11dn1. The enzyme significantly differs by its properties (specific activity, molecular mass, temperature optimum) from assimilatory enzyme [100].

Japanese scientists have identified a relationship between activity of NRase found in mitochondrial fraction of the fungus *F. oxysporum* MT-811 and ATP synthesis and also solubilized and partially purified the enzyme [99, 101]. Intact mitochondria of *F. oxysporum* exhibited NRase activity with physiological respiratory substrates (malate + pyruvate, succinate, formate) and with syn-

thetic electron donors (reduced methyl viologen, MVH). Detergent treatment of mitochondrial fraction resulted in a loss of NRase activity in the presence of respiratory substrates, but had no effect on the MVH-dependent enzyme activity. These results confirm the supposition concerning the NRase relationship with the respiratory chain. Besides, fungal NRase exhibited similarity by spectral characteristics, molecular mass, and cofactor to dissimilatory NRase of *E. coli* and other denitrifying bacteria containing molybdopterin, cytochrome *b*, and Fe-S proteins.

ALTERATIONS OF NITRATE REDUCTASE PROPERTIES AND STRUCTURE CAUSED BY STRESS FACTORS

At this stage of investigations properties and structure of NRases of organisms (microorganisms, plants) grown under stress conditions are still poorly studied. Nevertheless, this information is extremely important both for revealing general mechanisms of adaptation to stress and for applied investigations with due regard to the role of NRase in nitrogen turnover in nature.

It is known that many hyperthermophilic (*Pyrobaculum aerophilum* [102], *Halobacterium denitrificans* [103]) and thermophilic (*Thermus thermophilus* [104], *Caldithrix abyssi* [105], *Thermovibrio ammonificans* [106]) bacteria are able to utilize nitrate as electron acceptor under anaerobic conditions. The process of the extremophile denitrification is studied insufficiently, but NRase of some archaeobacteria (*P. aerophilum* [107], *Haloarcula marismortui* [108]) and certain *Haloferax* species [109] has been studied.

Nitrate reductase of *P. aerophilum* is structurally similar to the enzyme of *Haloferax volcanii* consisting of three subunits of 100, 60, and 31 kD [110] and is a membrane-bound enzyme, whereas NRases of *Haloferax denitrificans*, *Haloferax mediterranei*, and *Haloarcula marismortui* are localized in the periplasm [108, 109]. There may be an additional protein that binds NRase to the cytoplasmic membrane, NRase of *H. marismortui*, evidently, differs from the rest enzymes because it is a homotetramer consisting of 63 kD subunits. All NRases of halophilic archaeobacteria, except *H. denitrificans*, require an increased salt concentration for revealing activity and structural stabilization, but the increase in salt concentration resulted in a shift of temperature optimum of the *H. mediterranei* enzyme activity towards higher temperatures.

Nitrate reductase of hyperthermophilic archaeobacterium *P. aerophilum* was isolated from cells grown under anaerobic conditions in the presence of nitrate and low amounts of tungsten and molybdenum. The isolated enzyme has been characterized [107, 110]. The enzyme contained molybdenum (0.8 mol/mol protein) in its

active center and was structurally similar to NRases of mesophilic organisms [30], but differed from them by unusually high specific activity (326 U/mg), measured at 75°C with benzyl viologen as an electron donor; the activity measured at 95°C was even higher (526 U/mg). Like other dissimilatory NRases of mesophilic organisms, the enzyme of *P. aerophilum* was also able to utilize chlorate as a substrate at a rate exceeding that of nitrate utilization. The NRase affinity to nitrate and chlorate 4–60 times higher than this is described for dissimilatory NRases of mesophilic organisms for which the K_m value is 2–3 mM [30].

It should be noted that anaerobic growth of this bacterium on the nitrate-containing medium required the presence of 0.7–1.5 μ M tungstate, which could not be replaced by molybdate [107]. In most prokaryotes and eukaryotes, tungsten is an antagonist of molybdenum and easily replaces the latter within inactive analogs of molybdenum-containing enzymes [111]. Owing to this, tungsten is often used as a tool for studying structure and properties of molybdenum enzymes [112]. The addition of 100 μ M molybdate to culture medium completely inhibited bacterial growth. At low tungstate concentrations (0.3 μ M and lower) nitrite accumulation in the growth medium was observed, which was prevented by tungsten addition in higher concentrations. The dependence of *P. aerophilum* growth on tungsten suggests the incorporation of tungsten-containing enzymes into metabolic pathways, but the key enzyme of nitrate dissimilation NRase contained molybdenum in its active center [110].

A similar tungsten effect was also observed in the salt-tolerant yeast *Rhodotorula glutinis* on a medium containing molybdenum and tungsten in equimolecular amounts (1 mM). Tungsten stimulated Mo-co synthesis and following increase in NRase activity, but tungsten was not found in NRase; its activity was revealed only at the level of Mo-co expression [113, 114].

During the past decades a number of tungsten-containing enzymes have been isolated from cells of thermophilic archaeobacteria. Among them there are formate dehydrogenase, aldehyde:ferredoxin oxidoreductase, formaldehyde:ferredoxin oxidoreductase, etc. containing tungsten in their active center and not able to replace it with molybdenum. At the same time a number of enzymes were found whose catalytic activity can be exhibited both with molybdenum and tungsten (formyl methanofuran dehydrogenase, trimethylamine-N-oxide reductase, etc.). Molybdopterin, able to coordinate both molybdenum and tungsten, is the active center of these enzymes [112].

Very recently two NRases, free of molybdenum and molybdenum cofactor, have been isolated from the cells of vanadate-reducing bacterium *Pseudomonas isachenhovi* growing under high content of vanadium (0.5 g/liter) [115]. The periplasmic NRase was a 55-kD monomer, containing vanadium, whereas the membrane-

bound NRase was a heterodimer of 130 and 67 kD subunits and no metal was found in the active center [115].

The reductase complex isolated from the dissimilatory iron-reducing bacterium *Geobacter metallireducens* did not contain molybdenum and molybdenum cofactor in the active center, but contained cytochrome *c* and was able to reduce both nitrate and nitrite [116].

In our laboratory a highly active NRase of 130–140 kD was isolated from cells of a halophilic denitrifying bacterium of the *Halomonas* genus, strain AGJ 1-3. The enzyme was purified to homogeneity, and it contained no molybdenum or molybdo-cofactor in the active center [117]. The enzyme was characterized by thermal stability and relative insensitivity to NaCl in high concentrations, had temperature optimum at 70–80°C, and exhibited polyreductase activity by reducing nitrates and other oxoanions.

The above-mentioned data from the literature allow us to conclude unambiguously that stress factors in culture medium have a substantial effect on structural and functional characteristics of NRases. Attempts to systematize leading mechanisms of the pro- and eukaryotic organisms' "responses" to the environmental stress conditions were not successful, probably due to not enough experimental data. However, analysis of data from the literature indicates that, as a rule, alterations take place at the structural level of the active center, which results in a shift of the enzyme activity optimum to the region of higher temperatures, an increase in thermal stability, and changes in substrate specificity towards polyfunctionality; changes also happen at the level of synthesis of specific molecules that prevent or decrease the effect of stress factors on the enzyme.

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