Biochemistry (Moscow), Vol. 70, No. 7, 2005, pp. 799-803. Translated from Biokhimiya, Vol. 70, No. 7, 2005, pp. 968-973.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM04-165, February 13, 2005.

Characterization of Molybdenum-Free Nitrate Reductase from Haloalkalophilic Bacterium *Halomonas* sp. Strain AGJ 1-3

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Received July 2, 2004
Revision received July 13, 2004

Abstract—Nitrate reductase from the haloalkalophilic denitrifying bacterium *Halomonas* sp. strain AGJ 1-3 was isolated and purified to homogeneity. The isolated enzyme belongs to a novel family of molybdenum-free nitrate reductases. It presents as a 130-140 kD monomeric protein with specific activity of 250 μmol/min per mg protein. The enzyme reduces not only nitrate, but also other anions, thus showing polyoxoanion reductase activity. Enzyme activity was maximal at pH 7.0 and 70-80°C.

Key words: *Halomonas* sp., nitrate reductase, denitrification, alkalophiles, soda lakes

Biological transformation of nitrogen compounds is of widespread occurrence in nature, and its study facilitates better understanding of biogeochemical nitrogen turnover, which is dependent on the activity of both prokaryotes and eukaryotes. The state of oxidation of nitrogen engaged in the biogeochemical nitrogen cycle varies from +5 to −3. Nitrogen in its oxidized form is generally present only in oxyanions (NO_3, NO_2, NO, N_2O), and therefore it should be reduced (up to −3) to be incorporated in the biologically active molecules. Organisms reduce nitrogen oxides either to derive energy required or, just the opposite, to dissipate "excessive" energy generated during fermentation [1, 2]. This is a multistep process involving several successive reactions. Nitrate reductase catalyzes the fist reductive step, i.e., the conversion of nitrate to nitrite.

For the more than 50-year history of studying mechanisms underlying nitrate reduction, a rich variety of nitrate reductases from diverse organisms has been isolated and characterized [3]. Despite their great variability in the form and intracellular location, they all contained in their active center molybdenum as the molybdenum cofactor [3-6].

However, these nitrate reductases were generally isolated from organisms inhabiting "normal" environments. Only a few papers are available on nitrate reductases from archaea living in the presence of high (up to 4 M) NaCl concentrations in hot springs [7, 8]. From these microorganisms, molybdenum-containing nitrate reductases with rather high (70-80°C) temperature optimum for nitrate reduction were isolated. Study of nitrate reduction in the extremophilic eubacteria has so far been almost totally ignored. The canonical nitrate reductase is unstable under various environmental stress factors, such as high salinity, very high (>9.0) or low (<3.0) pH values, temperature, and heavy metals. Under these extreme conditions, the enzyme is disrupted, e.g., under salt stress [9], or looses its activity totally or significantly. For example, tungsten induces synthesis of the apoprotein, while the enzyme is either catalytically inactive, or its activity is very low [10]. Toxic metals or other stress factors can induce the replacement of molybdenum in the active enzyme center by other metals or trigger the synthesis of isofunctional enzymes encoded by distinct genes. From the vanadate-reducing bacterium *Pseudomonas isachenkovii* we have isolated two catalytically distinct molybdenum- and molybdocofactor-free dissimilatory nitrate reductases (periplasmic and membrane-bound, respectively) [11], which suggests the occurrence of "alternative" nitrate reductases lacking molybdocofactor in their active centers.

The goal of this study was to investigate a nitrate reductase from the haloalkalophilic denitrifying bacterium *Halomonas* sp. strain AGJ 1-3 isolated from the Naiwasha soda lake in Kenya.

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0006-2979/05/7007-0799

MATERIALS AND METHODS

Chemicals. Acrylamide, glycine, ammonium persulfate, tetramethylethylenediamine, triphenyltetrazolium chloride, β-galactosidase, phosphorylase *b*, bovine serum albumin, egg albumin, carboanhydrase, methyl viologen, and N-(1-naphthyl)ethylenediamine were purchased from Sigma (USA); sodium dithionite from Merck (Germany); Tris, bis-acrylamide, 2-mercaptoethanol, and NaN_3 from Fluka (Switzerland); KCN and Coomassie Brilliant Blue G-250 from Ferak (Germany); Toyopearl HW-55F from Toyo Soda (Japan). All other chemicals of analytical grade were obtained from domestic suppliers.

Organism and growth conditions. In this study we used the haloalkalophilic denitrifying bacterium *Halomonas* sp. strain AGJ 1-3 obtained as a pure isolate from the sediments of Naiwasha soda lake (Kenya). This is a moderately halophilic, obligately alkalophilic, actively denitrifying bacterium, optimally growing at pH 10.5 in the presence of 0.5 M Na^+. According to the taxonomic analysis, the bacterium was assigned to the a cluster of halophilic γ-proteobacteria of the genus *Halomonas*, closely related to *H. disederata* [12, 13].

Cells were routinely grown in medium containing (g/liter): Na_2CO_3, 20; $NaHCO_3$, 10; NaCl, 5; K_2HPO_4, 1; KNO_3, 1; $MgSO_4 \cdot 7H_2O$, 0.1; yeast extract, 0.2; $CH_3COONa \cdot 3H_2O$, 1; $Na_2S_2O_3 \cdot 5H_2O$, 0.25; pH 10.5, supplemented with microelements (1 ml/liter) prepared according to [14].

Cells grown anaerobically for 2-3 days at 30°C in 5-liter bottles were collected by centrifugation at 7000*g* for 15 min at 4°C, washed with a fresh portion of the cultivation medium, and stored at −20°C.

Preparation of cell extract. The frozen cells were thawed, suspended in 25 mM sodium phosphate buffer, pH 7.0, and disrupted in a French press at 200-220 atm. The homogenate was centrifuged at 15,000*g* for 30 min and the supernatant (the cell-free extract) was used as a source for nitrate reductase purification.

Nitrate reductase purification. Nitrate reductase was purified using non-denaturing preparative electrophoresis in 7.5% polyacrylamide gel with constant buffer elution. The column was eluted at a rate of 0.5 ml/min with fraction volume of 7.5 ml, and current intensities of 40 mA. The electrode and eluting buffer used was 50 mM Tris-H_3BO_3, pH 8.7.

Assay of nitrate reductase activity. Nitrate reductase activity was assayed with dithionite-reduced methyl viologen as the electron donor. The incubation medium contained 100 mM potassium phosphate buffer, pH 7.0, 10 mM KNO_3, 1.2 mM sodium dithionite, 0.8 mM methyl viologen, and 10-100-μl aliquots of enzyme preparations. The reaction was initiated by the addition of dithionite, allowed to proceed for 15 min at various temperatures, and terminated by the addition of a mixture containing 500 μl of 0.6% sulfanilic acid in 20% HCl and 500 μl of 2 mM N-(1-naphthyl)ethylenediamine. The absorbance at 548 nm was measured with a Spekol 11 spectrophotometer (Carl Zeiss, Germany) after 15 min required for color development.

Determination of molybdenum cofactor relied on the use of *Neurospora crassa* nit-1 mutant bearing nitrate reductase with defective molybdenum cofactor. Complementation of molybdenum cofactor with nit-1 mutant aponitrate-reductase is accompanied by assembling of the functionally active enzyme. Growth conditions of *N. crassa* nit-1 mutant and cell-free extract preparation were described earlier [15]. Molybdenum cofactor was determined as described in [16]. To ensure molybdenum cofactor dissociation, aliquots of the homogenous nitrate reductase were heated at 90°C for 90 sec in the presence of 2 mM reduced glutathione or 2 mM ascorbic acid as molybdenum cofactor protectors. Then the treated preparations were incubated at 5°C for 24 h in the presence of 2 mM sodium molybdate and 100 μl of the cell-free extract from *N. crassa* nit-1. After complementation, activity of the resulting nitrate reductase was determined with NADPH as the electron donor.

To determine pH optimum of the enzyme, the following buffer systems were used: 0.2 M CH_3COONa-CH_3COOH (pH 4-5), 0.2 M KH_2PO_4-KOH (pH 6-8), 0.2 M H_3BO_3-KCl-KOH (pH 9-10), and 0.2 M K_2HPO_4-KOH (pH 11-12). Activity of the enzyme at different pH values was determined at least three times.

Effect of Na^+ on enzyme activity was assayed by varying NaCl concentrations from 0 to 4.5 M.

Temperature optimum of the enzyme was determined by three measurements at each chosen temperature within the 30-100°C range.

Non-denaturing polyacrylamide gel electrophoresis (native PAGE) was carried out using a linear (5-15%) gel gradient in the plate (11 × 11 cm, 1-mm thick) in Tris-HCl, pH 8.8, by the method of Davis [17].

SDS-PAGE with a 5-20% linear gel gradient was performed according to the Laemmli method [18]. To determine the molecular masses of the enzyme subunits we used the following calibration standards (with their molecular masses): β-galactosidase (116 kD), phosphorylase *b* (97 kD), bovine serum albumin (67 kD), egg albumin (45 kD), and carboanhydrase (29 kD). Proteins resolved by SDS-PAGE were visualized with silver by the method of Nesterenko [19].

Enzyme activity in polyacrylamide gel was determined by submerging the gel in the reaction mixture containing 0.1 M sodium phosphate buffer, pH 6.8, 10 mM KNO_3 (or 1 mM $NaNO_2$, 5 mM $KClO_3$), 1 mM methyl viologen, and 5 mM sodium dithionite. The gel was incubated at 70°C until transparent lanes appeared against the blue background as a result of the enzyme-induced oxidation of methyl viologen. The gel was prefixed with 0.05%

triphenyltetrazolium chloride and finally fixed in 5% acetic acid.

Metal determination. Metal content in the homogenous nitrate reductase was analyzed by flame atomic emission spectrometry and flame mass-spectrometry with an Optima 2000 DV and ELAN 9000 (Perkin Elmer, USA), respectively.

RESULTS AND DISCUSSION

Cultivation of *Halomonas* sp. strain AGJ 1-3 under anaerobic conditions in the presence of 1 mM tungsten did not inhibit the denitrification process, suggesting either tolerance of the cells to tungsten, as in the case of *Pyrobaculum aerophilum* [20], or synthesis of the "alternative" nitrate reductase.

Electrophoretically homogenous nitrate reductase was obtained from the cell-free extract of *Halomonas* sp. strain AGJ 1-3 using preparative non-denaturing electrophoresis (Fig. 1a). The enzyme, based on SDS-PAGE data, is a 130 kD monomeric protein (Fig. 1b). Because nitrate, a nutrient component, is the sole electron acceptor in the respiratory chain, it seems reasonable to suggest that the isolated nitrate reductase falls into the class of dissimilatory nitrate reductases involved in energy production for cell life. These enzymes are generally located in the membrane and are composed of three subunit types, i.e., α (catalytic), β (electron-transporting), and γ (anchoring) [6]. Upon cell disruption, the isolated nitrate reductase was revealed in the soluble cellular fraction. Although we have no convincing evidence for the proper intracellular location of the isolated nitrate reductase, it seems likely that it can be located on the membrane surface, easily dissociating from the membrane into the soluble fraction during cell disruption.

Another distinctive property of the isolated nitrate reductase is that it is a monomeric form. Catalytic activity of the individual subunits of molybdenum-containing nitrate reductases has not been yet described. We have pioneered in describing catalytic activity of monomeric molybdenum-free nitrate reductases [11, 21, 22]. An ability of the nitrate reductase from *Halomonas* sp. strain AGJ 1-3 to reduce chlorate also indicates that the enzyme belongs to the class of dissimilatory "respiratory" nitrate reductases, although it is located in the soluble cellular fraction. Dissimilatory nitrate reductases located in the periplasmic space do not reduce chlorate [6]. However, the process of denitrification in alkalophiles has not been adequately investigated; therefore, it is possible that nitrate reductases of alkalophiles constitute a particular enzyme class. Conclusive proof of this suggestion requires more thorough studies.

Specific activity of homogenous nitrate reductase preparations attaining 250 μmol NO_2^- formed for min per mg protein is rather high compared to that of "classical

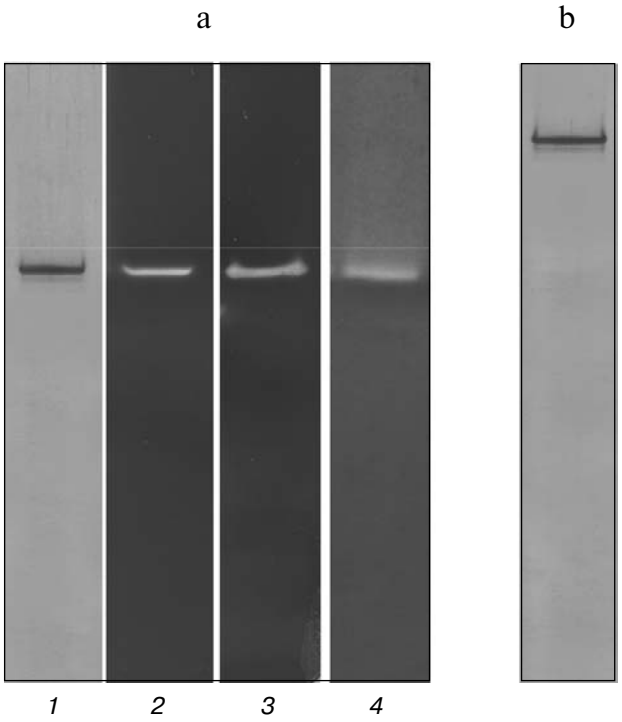

Fig. 1. Native (a) and denaturing SDS-PAGE (b) of the nitrate reductase purified from *Halomonas* sp. strain AGJ 1-3 using a two-step preparative electrophoresis under non-denaturing conditions (see "Materials and Methods"). Proteins resolved were visualized with silver (*1*) after incubation with nitrate (*2*), chlorate (*3*), or nitrite (*4*).

denitrifiers". For example, specific activity of the homogenous nitrate reductase from *Pseudomonas aeruginosa* ranged up to 2.8, for *Pseudomonas stutzeri* to 27, and for *Paracoccus denitrificans* to 50 μmol/min per mg protein, respectively [6]. The specific activity of the isolated nitrate reductase is second only to that of the nitrate reductase from hyperthermophilic archaea *Pyrobaculum aerophilum* – 326 (measured at 70°C) and 526 (measured at 95°C) μmol/min per mg protein [8].

The test for the presence of molybdenum cofactor in the isolated nitrate reductase using complementation with the nitrate reductase from nit-1 mutant of *N. crassa* bearing a defective molybdenum cofactor was negative, thus indicating that the enzyme is molybdenum-free. Direct measurements of metals using flame atomic emission spectrometry and flame mass-spectrometry also showed the absence of molybdenum in the enzyme. Azide and cyanide, metal-chelating agents, potently inhibited the activity of the enzyme, a half-maximal inhibition ($[I]_{0.5}$) was reached at 36 and 110 μM, respectively, which is typical for many metal-containing enzymes. According to our preliminary results, the prevailing metal was iron. Thus, the nitrate reductase isolated from the AGJ 1-3 strain belongs to the class of molybdenum-free alternative nitrate reductases.

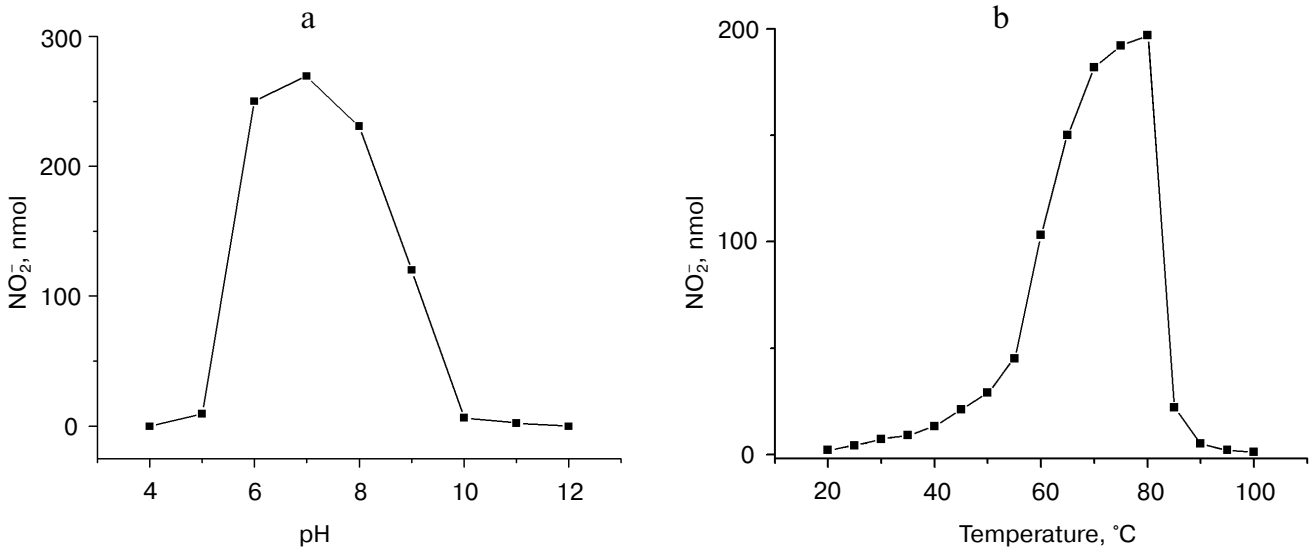

Fig. 2. Dependence of activity of the nitrate reductase from *Halomonas* sp. strain AGJ 1-3 on pH (a) and temperature (b).

Despite the highly alkaline nature of the growth culture, activity of the isolated nitrate reductase was maximal at pH 7.0 (Fig. 2a), which matches the pH optimum of most intracellular enzymes and corroborates with its intracellular location. It is well known [23] that under extreme environmental conditions protective barriers are confined not only to the cytoplasmic membrane harboring Na^+/H^+- and K^+/H^+-antiporter systems, which are indispensable for maintaining neutral or slightly alkaline (7.0-8.5) intracellular pH values, but also the cell wall containing components enriched with negatively charged carboxyl groups that repulse hydroxyl groups and absorb protons and sodium ions.

The temperature optimum for nitrate reductase activity was 70-80°C (Fig. 2b). The temperature optimum of most molybdenum-containing nitrate reductases varies from 30 to 40°C, while for enzymes from archaea it ranges from 60 to 80°C, for nitrate reductase from hyperthermal archaea *Pyrobaculum aerophilum* it reaches about 100°C [8]. Interestingly, the halophilic bacterium *Halomonas* sp. AGJ 1-3 isolated from the soda lake and assigned to the *Halomonas* genus is a mesophilic one, so the observed rather high temperature optimum is unusual for enzymes isolated from mesophilic organisms [24]. However, the elevated temperature optimum is typical for all so far isolated molybdenum-free nitrate reductases and may be considered as their hallmark [21, 22].

The isolated enzyme was rather thermostable compared to other nitrate reductases from bacterial denitrifiers. Nitrate reductase activity remained constant when the enzyme was heated at 50°C for 100 min, while at 70°C it lost half of its activity in 35 min. A relative high thermostability has already been intimated for the class of molybdenum-free nitrate reductases [20]. The reasons for high thermostability and elevated temperature optimum of molybdenum-free nitrate reductases are still unclear. The bacterium under study was isolated from the Kenyan soda lake exposed to constant high insolation and hence, constant temperature stress. Therefore, it seems reasonably to suggest that high thermostability and elevated temperature optimum of the enzyme is a reflection of the organism's adaptation to temperature stress conditions.

The enzyme was rather insensitive to high NaCl concentrations. In the presence of 0.5-3.5 M NaCl, the enzyme retained approximately 60% of the initial activity. Activity declined to 40% when NaCl concentration was increased to 4.5 M.

We have already mentioned that the isolated nitrate reductase reduced not only nitrate, but also chlorate and nitrite, thus possessing polyreductase activity (Fig. 1a). However, its activity with nitrite as a substrate was not so pronounced compared with nitrate and chlorate, attaining only approximately 5% from nitrate reductase activity; moreover, nitrite reductase activity was observed only when nitrate was omitted.

The ability to reduce several anions was originally shown for the heme *c*-containing membrane-bound enzyme complex from the iron-reducing bacterium *Geobacter metallireducens* [25]. The molybdenum-free enzyme complex reduced nitrate, nitrite, chlorate, selenate, fumarate, and thiosulfate. A molybdenum-free periplasmic nitrate reductase from the alkalophilic bacterium *Thioalkalivibrio nitratreducens* also reduced nitrate, nitrite, chlorate, bromate, sulfite, and selenate [22].

Reduction of each anion is generally catalyzed by a specific reductase with pronounced substrate specificity.

However, our and literature data suggest the existence of an enzyme group with polyreductase activity. It is conceivable that the broad substrate specificity is a mechanism underlying the organism's adaptation to extreme conditions of natural habitats. *T. nitratreducens* and *Halomonas* sp. were isolated from soda lakes containing a wide variety of cations and anions.

Thus, from the haloalkalophilic denitrifying bacterium *Halomonas* sp. AGJ 1-3 we isolated and purified to homogeneity nitrate reductase with molecular mass of 130 kD. The enzyme belongs to the novel class of molybdenum-free nitrate reductases. It is a high-capacity, thermostable enzyme, relatively tolerant to high NaCl concentrations, present in a monomeric form, and reduces not only nitrate but also other oxyanions, thus displaying polyreductase activity. Enzyme activity was maximal at pH 7.0 and 70-80°C, which, as have been already shown by us [21, 22], is typical for molybdenum-free nitrate reductases.

Understanding of mechanisms underlying nitrate reduction in different organisms, especially in extremophiles, is of both fundamental and applied importance.

Extremophiles can be applied as biological components for remediation of various technogenic environments. In particular, alkaliphilic denitrifying microorganisms can be used as a waste-removal system to eliminate excessive amounts of nitrates from alkaline industrial wastes.

This study was supported by the Russian Foundation for Basic Research (grants 04-04-49112 and 04-04-48647), by INTAS (grant 03-55-1721), and by the Russian Academy of Sciences (grant on cellular and molecular biology).

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