ISSN 0006-2979, Biochemistry (Moscow), 2010, Vol. 75, No. 6, pp. 744-751. © Pleiades Publishing, Ltd., 2010.
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# Isolation and Characterization of Nitrate Reductase from the Halophilic Sulfur-Oxidizing Bacterium *Thioalkalivibrio nitratireducens*

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Received September 1, 2009
Revision received October 28, 2009

**Abstract**—A novel nitrate reductase (NR) was isolated from cell extract of the haloalkaliphilic bacterium *Thioalkalivibrio nitratireducens* strain ALEN 2 and characterized. This enzyme is a classical nitrate reductase containing molybdopterin cofactor in the active site and at least one iron–sulfur cluster per subunit. Mass spectrometric analysis showed high homology of NR with the catalytic subunit NarG of the membrane nitrate reductase from the moderately halophilic bacterium *Halomonas halodenitrificans*. In solution, NR exists as a monomer with a molecular weight of 130-140 kDa and as a homotetramer of about 600 kDa. The specific nitrate reductase activity of NR is 12 μmol/min per mg protein, the maximal values being observed within the neutral range of pH. Like other membrane nitrate reductases, NR reduces chlorate and is inhibited by azide and cyanide. It exhibits a higher thermal stability than most mesophilic enzymes.

**DOI**: 10.1134/S000629791006009X

*Key words*: *Thioalkalivibrio nitratireducens*, haloalkaliphilic bacteria, nitrate reductase, NarG

Nitrogen is ubiquitous in living organisms. Since oxidation degree of nitrogen varies from +5 to –3, the nitrogen cycle in nature is a complex, multistage process including a cascade of oxidative (nitrification) and reducing (denitrification, dissimilatory nitrate reduction) reactions. The first reaction of the oxidative cycle is catalyzed by nitrate reductases (NR) that reduce nitrate to nitrite by a two-electron mechanism [1, 2].

Three classes of nitrate reductases have been found in prokaryotes: assimilatory nitrate reductases (Nas) and two classes of dissimilatory enzymes (respiratory membrane-bound nitrate reductases (Nar) and periplasmic nitrate reductases (Nap)). In spite of the structural differences and various localization in the cell, all known nitrate reductases contain a molybdenum cofactor (Moco) in the active site as the molybdopterin guanine dinucleotide (MGD) [3, 4]. Besides MGD, nitrate reductase complexes contain various prosthetic groups involved in the electron transport from a donor molecule to the active site of the enzyme (FeS clusters, cytochromes *b* and *c*, FAD) [2, 4, 5].

Nas are soluble enzymes localized in the cytoplasm. They can be ferredoxin (flavodoxin)- or NADH-dependent. The ferredoxin-dependent Nas consists of one catalytic subunit (70-85 kDa) [6]. The NADH-dependent Nas is a heterodimer containing a catalytic subunit (90-105 kDa) and a subunit that is responsible for the electron transport (45-50 kDa) [7]. Both types of the enzymes contain MGD and FeS clusters. The synthesis of Nas is oxygen-insensitive, inhibited by ammonium, and induced by nitrate and nitrite [8, 9].

Nar play a key role in the formation of the transmembrane proton gradient [4]. The Nar molecule is composed of three subunits – NarG, NarH, and NarI. Subunits NarG and NarH are localized in the cytoplasm, and the NarI subunit is a membrane anchor. The catalytic NarG (104-150 kDa) contains a molybdenum cofactor and a [4Fe-4S] cluster, NarH (43-63 kDa) is a globular

*Abbreviations*: HPLC, high performance liquid chromatography; MGD, molybdopterin guanine dinucleotide; Nap, periplasmic dissimilatory nitrate reductases; Nar, respiratory membrane-bound nitrate reductases; NarGHI, membrane-bound nitrate reductase composed of NarG, NarH, and NarI subunits; Nas, assimilatory nitrate reductase; NR, nitrate reductase from *Thioalkalivibrio nitratireducens*; PPB, potassium-phosphate buffer.

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protein containing iron–sulfur clusters [10], and NarI (19-28 kDa) contains two *b*-type hemes. Besides the reduction of nitrates, enzymes of this class can reduce chlorates, and they are inhibited by cyanide and azide [11].

Nap play a key role under both aerobic and anaerobic metabolism in fixation of trace amounts of nitrite and elimination of excess of reducing equivalents and are not involved in the formation of the transmembrane potential. These enzymes are localized in the periplasm. They are heterodimers composed of a catalytic subunit (90 kDa) containing a molybdenum cofactor and a [4Fe-4S] cluster, and a subunit of 13-19 kDa containing two *c*-type hemes. Nap do not use chlorate as a substrate, they are insensitive to cyanide, and they are activated in the presence of azide and thiocyanate [11]. Nap synthesis is insensitive to oxygen and ammonium and is stimulated by the addition of nitrate [12].

The bacterium *Thioalkalivibrio nitratireducens* strain ALEN 2 was isolated from sediment of the hypersaline soda lake Fazda (Wadi Natrun) in Egypt. It is a haloalkaliphile with obligate chemolithoautotrophic metabolism and is capable of growing with sulfide/polysulfide and thiosulfate as electron donors under both aerobic and anaerobic conditions at the expense of nitrate reduction. In the latter case, nitrate is stoichiometrically reduced to nitrite [13]. Previously, this bacterium was used for isolation of an octaheme cytochrome *c* nitrite reductase that significantly differed from earlier described cytochrome *c* nitrite reductases in its structure and properties. The enzyme exhibited high activity *in vitro* and was extremely thermostable [14, 15]. Presumably, the unique properties of the nitrite reductase reflect adaptation of *Tv. nitratireducens* to extreme environmental conditions.

The goal of the present work was isolation and characterization of nitrate reductase (NR) from *Tv. nitratireducens* ALEN 2 grown under microaerophilic conditions.

## MATERIALS AND METHODS

In the present work the following chemicals were used: acrylamide, glycine, tetramethylethylenediamine, bisacrylamide, 2-mercaptoethanol, triphenyltetrazolium chloride, methyl viologen, N-1-naphthyl ethylenediamine, NADH, phenylmethylsulfonyl fluoride, Tris, potassium dihydrophosphate, Coomassie G-250, and Triton X-100 (Sigma, USA); sodium dithionite (Merck, Germany; Aldrich, USA). Other chemicals were purchased from Reachim (Russia).

**Culture and growth conditions.** The culture of *Tv. nitratireducens* strain ALEN 2 was maintained as described earlier [14]. Cells were grown at 28°C under microaerophilic conditions. The mineral medium (pH 10.0) contained (g/liter): $Na_2CO_3$, 23; $NaHCO_3$, 7; NaCl, 5; $K_2HPO_4$, 1; $KNO_3$, 1; $MgCl_2 \cdot 6H_2O$, 0.2, and a number of microelements [16]. Thiosulfate (40 mM) was used as the source of energy. The inoculate was grown in the same medium supplemented with 5 mM $NH_4Cl$ in 750-ml flasks (working volume, 500 ml) on rotor shaker (220 rpm). Maximal nitrate reductase activity was found in exponentially growing cells. At this growth stage, cells were collected by centrifugation (13,000*g*, 30 min) and broken using a French press in 100 mM potassium-phosphate buffer (PPB), pH 7.0, in the presence of 0.2 mM phenylmethylsulfonyl fluoride (a serine proteinase inhibitor). Unbroken cells and cell debris were removed by centrifugation (13,000*g*, 30 min). To isolate cell membrane fractions, the resulting extract was centrifuged at 150,000*g* for 1.5 h. The membrane fraction was washed twice with 50 mM PPB, pH 7.0, and membrane proteins were solubilized using Triton X-100 (final concentration, 2% (v/v)) at 4°C overnight. The resulting solution was assayed for nitrate and nitrite reductase activity using the standard procedure described below. The soluble fraction was assayed for nitrite reductase activity using the same method. The nitrate reductase activity of the soluble fraction was revealed by gel staining, since high nitrite reductase activity prevented the quantitative determination of nitrate reductase activity in the solution.

**Isolation and purification of nitrate reductase (NR).** NR was isolated from the soluble fraction of the cell extract. The procedure of enzyme purification included ammonium sulfate fractionation, anion-exchange low-pressure chromatography, anion-exchange HPLC, and gel filtration.

Anion-exchange low-pressure chromatography was performed at 4°C using a DEAE-Sepharose (Fast Flow) column (80 ml) and a BioLogic LP chromatograph (Bio-Rad, USA). The column was equilibrated with 50 mM PPB, pH 7.0. After loading the extract onto the column and subsequent washing, the protein was eluted with a linear (0-0.6 M) NaCl gradient in the same buffer. Fraction volume was 7 ml. The elution of the enzyme was monitored by measuring activity and absorbance at 280 nm ($A_{280}$). Fractions exhibiting nitrate reductase activity were pooled, dialyzed against 50 mM PPB, pH 7.0, and applied onto the column for repeated anion-exchange chromatography.

Anion-exchange HPLC was performed using an AKTA FPLC chromatograph (Amersham Biosciences, USA) on a MonoQ 10/100 GL column equilibrated with 50 mM PPB, pH 7.0. Nitrate reductase was eluted with a linear (0-0.6 M) NaCl gradient in the same buffer.

Gel filtration was performed using the same chromatograph on a Superdex$^{TM}$ 200 10/300 column equilibrated with 50 mM PPB, pH 7.0, containing 0.15 M NaCl. Molecular weights of the eluted proteins were determined using protein standards (Amersham Biosciences): thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), chymotrypsinogen (25 kDa), and ribonuclease (13.5 kDa).

Blue Dextran (2000 kDa) was used to determine the void volume of the column.

**SDS-PAGE** was performed according to Laemmli [17] in a gradient (5-20%) polyacrylamide gel. Protein bands were stained with silver according to [18] and colloid Coomassie G-250. Molecular weights of proteins were estimated using a kit of recombinant proteins of 10, 15, 20, 25, 30, 40, 50, 60, 70, 85, 100, 120, 150, and 200 kDa (Fermentas, Lithuania).

**Mass spectra** were obtained using a Bruker Ultraflex II MALDI-TOF mass spectrometer equipped with a UV laser. Protein samples were preliminarily hydrolyzed by trypsin. The mass spectra of the resulting peptides were obtained in the positive ion reflectron mode. The accuracy of the measured masses after additional calibration by trypsin autolysis peaks was held to within 0.01%.

The peptide fingerprints of the proteins were identified using the Mascot program (www.matrixscience.com). The proteins were searched among bacterial proteins in the NCBI database with the indicated accuracy considering the possibility of methionine oxidation by oxygen and possible modification of cysteines by acrylamide.

**Determination of nitrate and nitrite reductase activities.** Nitrate reductase activity was determined by the rate of the formation of nitrite from nitrate in the presence of methyl viologen reduced with dithionite. The reaction was performed under anaerobic conditions in glass vials for gas chromatography. The reaction medium and a sodium dithionite solution were purged with argon of high purity for 10 min. The reaction medium (1 ml) contained 100 mM PPB, pH 7.0, 2 mM $NaNO_3$, 1.6 mM methyl viologen, and the tested enzyme (10-50 μl of 0.2-9 mg/ml solution). The reaction was started by the addition of 15 μl of 1 M sodium dithionite.

During 15-20 min of incubation at 30°C, aliquots of 50 μl were taken from the mixture to determine the nitrite concentration according to [19]. The unit of NR activity was determined as the amount of enzyme catalyzing formation of 1 μmol $NO_2^-$ per minute. All measurements were performed three times. Kinetic parameters of the nitrate reductase reaction were determined by varying the concentration of nitrate in the reaction mixture.

The nitrite reductase activity was determined by the rate of decay of nitrite under the same conditions, replacing 2 mM $NaNO_3$ in the reaction mixture with 1 mM $NaNO_2$ and monitoring the residual nitrite concentration.

**Determination of nitrate (nitrite, chlorate) reductase activities in polyacrylamide gels.** Non-denaturing electrophoresis was performed following Davis [20] using a gradient (5-20%) polyacrylamide gel containing 2 M urea. The gel was stained with colloidal Coomassie G-250.

To determine reductase activities, the gel was placed into a mixture containing 80 mM PPB, pH 7.0, 20 mM nitrate (nitrite, chlorate), 1 mM methyl viologen, and 5 mM sodium dithionite, and incubated at 45°C until the emergence of transparent bands on the blue field that are formed as a result of oxidation of the reduced methyl viologen by NR or nitrite reductase. To fix the staining, the gel was placed first in 0.05% triphenyltetrazolium chloride solution and then into 5% acetic acid.

**Optimal pH range.** To determine the optimal pH for NR activity, the following buffers 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 $H_3BO_3/KOH$ (pH 9-10), 0.2 M $K_2HPO_4/KOH$ (pH 11-12).

**Optimal temperature for NR activity** was determined after 10 min incubation of the reaction mixture at a certain temperature (20-70°C), and then the reaction was initiated by the addition of sodium dithionite. Other conditions were standard.

**Thermoinactivation of the enzyme.** Enzyme solution in 50 mM PBB, pH 7.0, was incubated at 40, 45, 50, or 55°C. After fixed time intervals, aliquots were taken from the mixture to determine the residual nitrate reductase activity at 30°C under standard conditions.

## RESULTS AND DISCUSSION

Analysis of solubilized proteins of the membrane fraction and proteins of the soluble fraction by non-denaturing PAGE revealed several bands exhibiting nitrate reductase activity that depended on changes in cultivation conditions (oxygen content, presence of ammonium and nitrite in the culture medium). In the present work, we describe the nitrate reductase from the soluble fraction of the cell extract whose synthesis was not inhibited either by oxygen or by ammonium.

Since in the soluble fraction a high nitrite reductase activity was revealed, it was impossible to evaluate the nitrate reductase activity. On the first stage of isolation, the nitrite- and nitrate reductases were separated by ammonium sulfate fractionation. NR was precipitated in the range of 20-40% sulfate ammonium saturation, while the nitrite reductase activity was detected in the solution until 60% saturation. The pellet exhibiting NR activity was dissolved in 50 mM PPB, pH 7.0, and applied to a DEAE-Sepharose column. The proteins were eluted with a NaCl (0-600 mM) gradient. The NR activity was observed in fractions with salt concentration of 210-450 mM (Fig. 1a and Fig. 3a, lane *4*). The subsequent chromatography of the obtained fraction on a MonoQ column yielded peaks I and II (Fig. 1b and Fig. 3a, lanes *5* and *6*) eluted with 260 and 350 mM NaCl, respectively. The peaks were collected separately and gel filtered through a Superdex 200 column equilibrated with 25 mM PPB containing 150 mM NaCl, pH 7.0.

According to the results of gel filtration, peaks I and II contained two fractions with NR activity with retention volumes of 9.46 and 12.6 ml (Fig. 2, a and b) correspon-

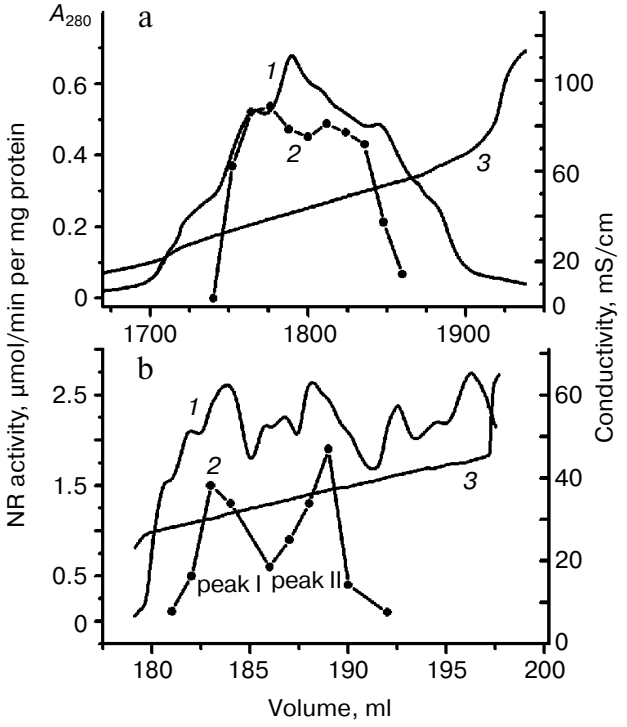


**Fig. 1.** NR purification by anion-exchange chromatography. a) DEAE-Sepharose chromatography; b) HPLC on a MonoQ column. *1*) Absorption at 280 nm; *2*) activity of NR, μmol $NO_2^-$/min; *3*) conductivity, mS/cm.

ding to molecular weights of 600 and 140 kDa. Peak I eluted at a lower NaCl concentration contained mainly the low-molecular-weight NR, while peak II contained the high-molecular-weight NR. Repeated gel filtration of the peaks with the retention volumes of 9.46 ml (Fig. 2c) and 12.6 ml (data not shown) yielded two NR preparations, NR1 and NR2. Non-denaturing electrophoresis of proteins NR1 and NR2 showed two closely spaced bands (Fig. 3b, lanes *1* and *2*) exhibiting NR activity (Fig. 3b, lanes *3* and *4*). Analysis of NR1 and NR2 by SDS-PAGE detected protein bands corresponding to 140 kDa (Fig. 3a, lanes *7* (NR2) and 8 (NR1)) kDa. The coinciding of all main peaks in the peptide mass spectra of the 140-kDa subunits of the two enzyme forms suggests that NR1 and NR2 are the same protein. The results of the mass-spectrometric assay revealed a high homology of the isolated protein with the catalytic NarG subunit of the membrane nitrate reductase from the moderately halophilic bacterium *Halomonas halodenitrificans*. In the isolated protein, polypeptide fragments were found that are conserved for the family of respiratory membrane nitrate reductases [21] and located in the domains that are responsible for binding MGD.

It has been known that the catalytic subunit NarG of respiratory membrane nitrate reductases is located on the cytoplasmic side of the cell membrane, and during the isolation it can readily dissociate from the membrane in a complex with the electron-transfer subunit NarH. The complex NarGH can be solubilized either by thermal treatment (40-60°C for 20 min) [22], or in the presence of detergents [23]. For the bacterium *Paracoccus denitrificans*, the soluble form of the complex was obtained by washing the membranes with 20 mM Tris buffer in the absence of $Mg^{2+}$ [24]. In some cases, as it was shown for nitrate reductase from the halophilic archaeon *Haloarcula marismortui* [23], the solubilization of the nitrate reductase complex resulted in the separation of the subunit NarG that reversibly aggregated in the solution yielding tetramers. A similar situation is likely to be observed in the present work, where two fractions NR1 and NR2 with different molecular weight are the tetrameric and monomeric forms of the same protein, the catalytic subunit NarG of the respiratory nitrate reductase. According to preliminary data, the oligomerization is reversible and is regulated by ionic strength.

The absorption spectra of oxidized forms of NR1 and NR2 exhibited a maximum at 408 nm that shifted to 418 nm after reduction with sodium dithionite (Fig. 4). The intensity of the peak decreased with the reduction, which is typical for iron–sulfur clusters [1, 7] found in the catalytic subunits of all types of nitrate reductases. The

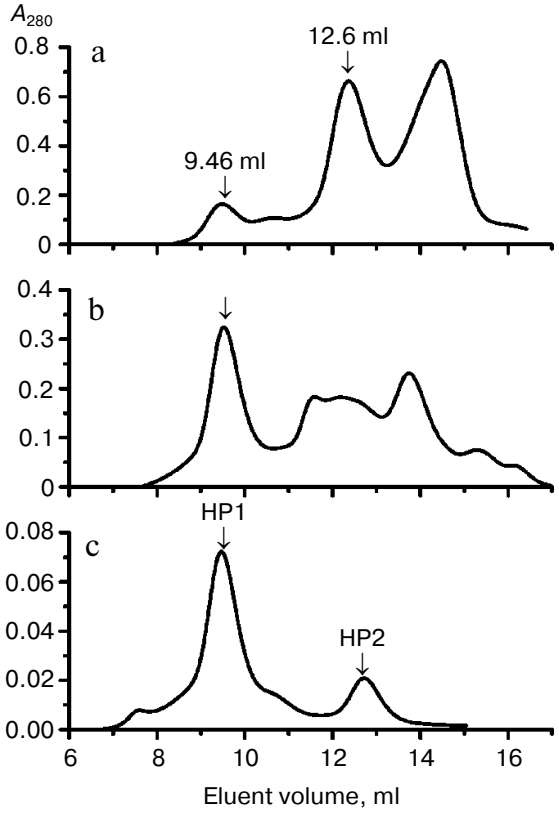


**Fig. 2.** Purification of NR by gel chromatography on a Superdex 200 column: a) peak I; b) peak II; c) repeated gel chromatography of the peak with the retention volume of 9.46 ml (see Fig. 2b).

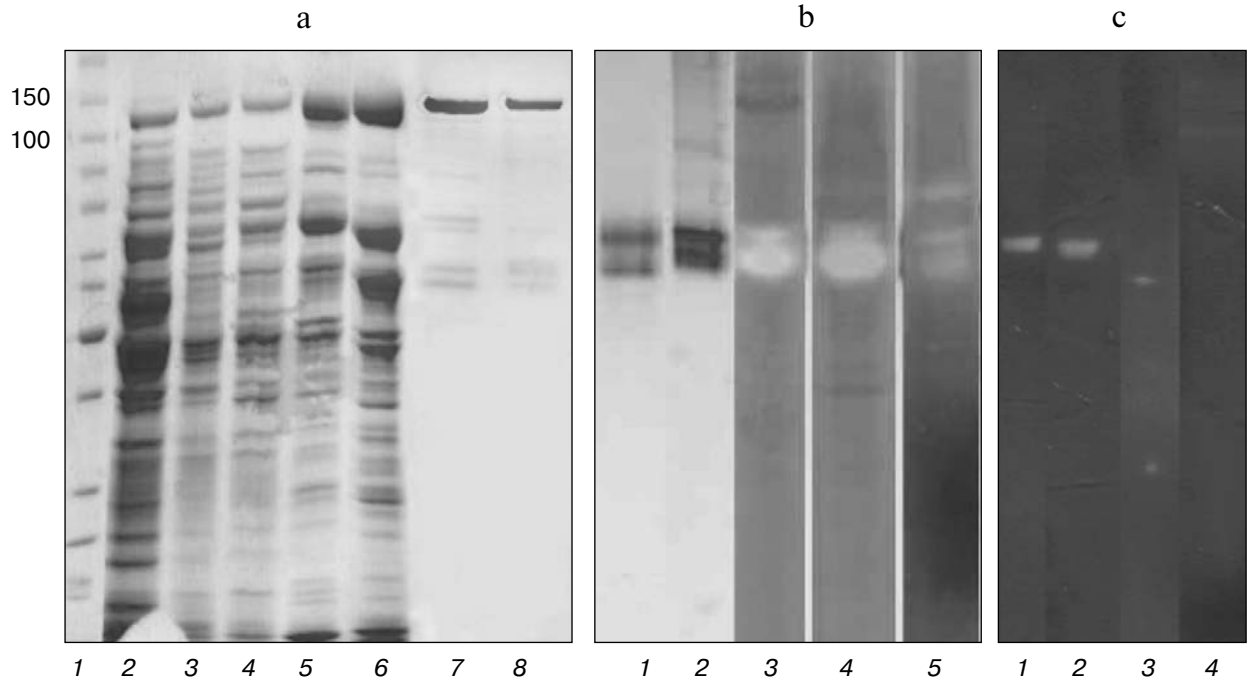


**Fig. 3.** Electrophoretic analysis of NR at different stages of purification. a) SDS-PAGE: *1*) protein standards; *2*) homogenate; *3*) fraction with nitrate reductase activity (20-40% saturation with ammonium sulfate); *4*) NR fraction after DEAE-Sepharose chromatography; *5*) purification on a MonoQ column, peak I; *6*) purification on a MonoQ column, peak II; *7*) NR2 fraction after gel filtration on Superdex 200; *8*) NR1 fraction after gel filtration on Superdex 200. b) Non-denaturing electrophoresis: *1*, *2*) NR1 (*1*) and NR2 (*2*) after Coomassie G-250 staining; *3-5*) NR1 (*3*), NR2 (*4*), and homogenate (*5*) after staining for nitrate reductase activity. c) Non-denaturing electrophoresis, staining for reductase activity with different substrates: *1*) nitrate; *2*) chlorate; *3*) nitrite; *4*) control (no substrates).

intensity of the NR1 and NR2 peaks calculated per unit of protein concentration differed only slightly. There were no peaks of cytochromes or flavins in the spectra of the proteins. Staining of polyacrylamide gels for detection of heme *c* in the bands corresponding to NR1 and NR2 was negative [25].

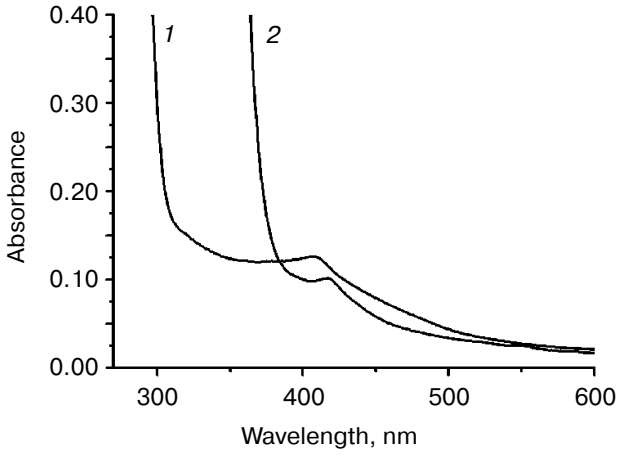


**Fig. 4.** Absorption spectra of the purified NR preparation: *1*) oxidized form; *2*) after reduction with dithionite. The sample contained 0.95 mg/ml of protein in 50 mM PPB, pH 7.0.

Direct determination of metals by plasma atomic-emission spectroscopy and plasma mass spectroscopy revealed the presence of iron and molybdenum atoms in both preparations ($5.3 \pm 0.3$ and $0.17 \pm 0.03$ mol per mol of 140-kDa subunit). Previously, it has been demonstrated that besides MGD, the catalytic subunits of all nitrate reductases contain one iron–sulfur cluster of [4Fe-4S] type. The content of iron atoms in the isolated NR corresponds to the presence of at least one [4Fe-4S] cluster. Some excess of iron can be related to the presence of iron-containing impurities (for example, NarH that contains four FeS clusters). The low molybdenum content in both preparations may be due to the loss of the metal during the isolation, as it has been described for other nitrate reductases [23].

EPR spectroscopy at 77 K showed no clear signals typical for Mo(V) (data not shown), presumably due to the low content of Mo in the active site of the NR. In spite of high content of iron, signals with g-factors of 1.8-2.2 that are characteristic for [4Fe-4S] clusters were not observed in the spectra. As known from the literature, in contrast to the catalytic subunits NasB and NapA, the signal of the [4Fe-4S]-cluster with g-factors in this range is not detected in the EPR spectra of the catalytic subunit NarG [26]. For the first time, the presence of this cluster in the NarG structure was demonstrated by X-ray analy-

sis of the complexes NarGHI and NarGH from *E. coli* [27, 28]. The signal with g-factors of 5.023 and 5.556 that was previously found [29] and attributed to the high-spin [4Fe-4S] cluster of the NarG subunit of the nitrate reductase from *E. coli* is sensitive to temperature and to the content of molybdenum in the active site and is virtually not detected at temperatures above 20 K and in the molybdenum-free NrGHI mutant.

To characterize catalytic properties of the enzyme, we used the NR1 preparation (further in the text, NR) since it was pure and obtained in sufficient amounts. Incubation of polyacrylamide gels obtained after non-denaturing electrophoresis of NR with different substrates showed that besides nitrate, NR reduced chlorate and did not reduce nitrite (Fig. 3c). The addition of 2 mM chlorate into the reaction mixture containing 2 mM nitrate (the concentration 10-fold exceeding the $K_m$ value for nitrate, see below) resulted in 20% decrease in nitrate reductase activity, confirming the effective binding of chlorate in the active site of NR. The ability to reduce chlorate is considered to be a characteristic feature of dissimilatory membrane-bound nitrate reductases [10, 30]. Periplasmic (Nap) and assimilatory (Nas) nitrate reductases do no reduce chlorate [2, 10, 31].

Specific nitrate reductase activity of NR at 30°C ranged up to 12 ± 2 μmol/min per mg protein (approximately 30 $sec^{-1}$). Change of the buffer (PPB or Tris-HCl, pH 7.5) did not influence the enzyme activity. The Michaelis constant value ($K_m$) for nitrate was 0.22 ± 0.05 mM. In spite of the low content of molybdenum in the preparation, the catalytic characteristics of the isolated NR are comparable with those determined for Nar and Nap enzymes from other sources: $K_m$ values for nitrate usually vary from 0.3 to 3.8 mM, and specific activity is within the range of 2.8-100 μmol/min per mg protein [2].

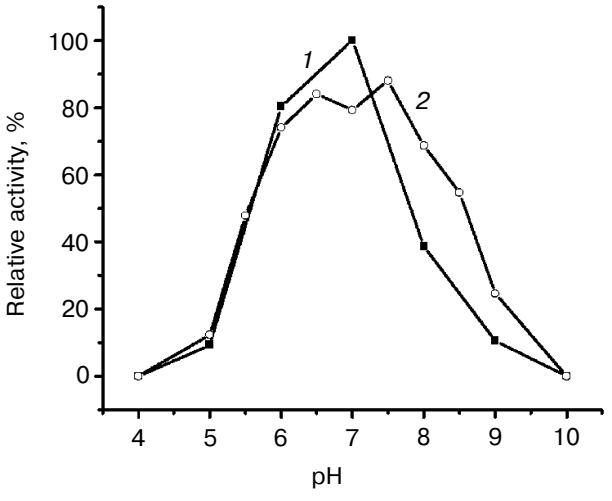


**Fig. 5.** Dependence of nitrate reductase activity on pH at 30 (*1*) and 45°C (*2*). The reaction mixture contained 4 μg/ml of NR in 100 mM PPB, pH 7.0.

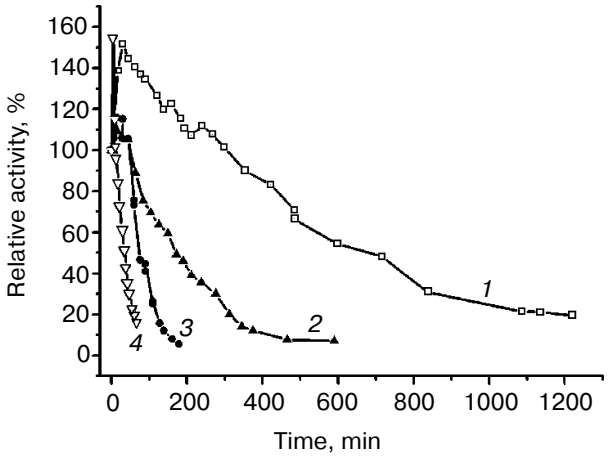


**Fig. 6.** Thermoinactivation of NR at 40, 45, 50, and 55°C (*1-4*, respectively). The reaction mixture contained 8 μg/ml of NR in 50 mM PPB, pH 7.0. The activity was determined at 30°C.

In spite of the alkaline environment and cultivation medium (pH 10) of the bacterium *Tv. nitratireducens*, the optimal pH value for the enzymatic activity of NR was close to the neutral one (pH 7.0 at 30°C and 6.5-7.5 at 45°C) (Fig. 5), thus corresponding to the pH optimum of most intracellular enzymes. In the presence of high salt concentrations, no significant change in the activity was observed. In the presence of 4.5 M NaCl, the activity constituted 70% of the original value.

The optimal temperature for the activity was found to be 50-55°C, which is higher than that of most bacterial nitrate reductases (30-40°C) [32]. Activity of the NR measured at the optimal temperature was equal to 40 μmol/min per mg protein.

Incubation of the enzyme solution at 40-55°C (50 mM PPB, pH 7.0) resulted in the activation of the enzyme by 20-60% for the first 30-40 min, and further the activity decreased to zero (Fig. 6). The thermoinactivation followed first order kinetics with constants of (0.3 ± 0.05)·$10^{-4}$, (1.0 ± 0.2)·$10^{-4}$, (3.5 ± 0.8)·$10^{-4}$, and (6.0 ± 1.0)·$10^{-4}$ $sec^{-1}$ at 40, 45, 50, and 55°C, respectively. The activation energy of the NR thermoinactivation calculated from these data was 174 kJ/mol, the value exceeding the corresponding parameter of mesophilic enzymes and indicating that the isolated enzyme belongs to the class of thermostable proteins, similarly to the previously isolated cytochrome *c* nitrite reductase isolated from the same bacterium [14].

According to literature data, the dissimilatory membrane nitrate reductases are competitively (towards nitrate) inhibited by cyanide, azide, and thiocyanate, and cyanide exhibits a higher affinity to the reduced form. For the periplasmic nitrate reductases, the inhibition by azide, cyanide, and thiocyanate was not observed [31]. NR isolated from *Tv. nitratireducens* was efficiently inhibited by cyanide or azide only when the reaction was initi-

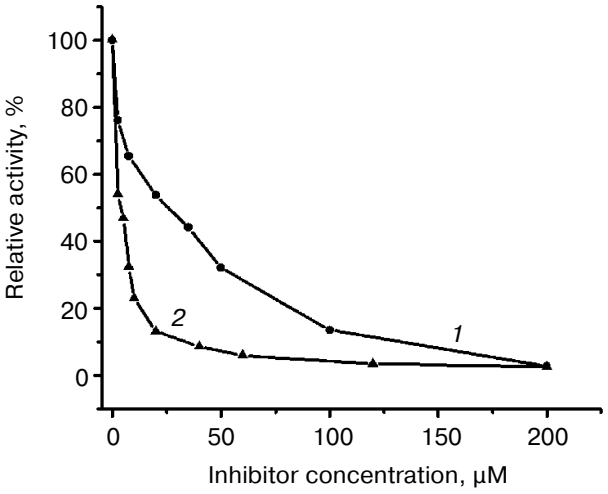


**Fig. 7.** Dependence of nitrate reductase activity on cyanide (*1*) and azide (*2*) concentrations. The reaction mixture contained 4 μg/ml of NR in 100 mM PPB, pH 7.0.

ated by the addition of nitrate to the reaction mixture containing the reduced enzyme and inhibitor. Under these conditions, azide was a more efficient inhibitor than cyanide: the inhibitor concentration resulting in 50% inhibition ($[I_{50}]$) was 3.4 μM for azide and 24.3 μM for cyanide (Fig. 7). The inhibition by azide was competitive towards nitrate with an inhibition constant of 0.3 μM, i.e. the binding of azide was 1000-fold more efficient than the binding of the substrate.

When the reaction was initiated by the addition of dithionite to the reaction mixture containing nitrate, the oxidized enzyme, and an inhibitor, the concentration of nitrite increased on the first stage due to the reduction of nitrate. After 10 min of incubation, the observed inhibition was proportional to the inhibitor concentration as in the case of the initiation of the reaction by nitrate. Thus, as in the case of membrane nitrate reductases, azide and cyanide exhibited a higher affinity to the reduced form of the enzyme.

Thus, in the present work NR was isolated from the haloalkaliphilic bacterium *Tv. nitratireducens* and characterized. In terms of the molecular and catalytic properties, NR was found to be similar to the catalytic subunit NarG of the membrane nitrate reductase. The factors responsible for the high content of NR in the soluble fraction of the cell extract are unclear. Presumably, besides respiratory functions of NR as a catalytic subunit in the membrane NarGHI complex, it can play a role of assimilatory nitrate reductase localized in the cytosol. To test this assumption, the investigation of the effect of cultivation conditions of *Tv. nitratireducens* cells on the NR synthesis will be continued.

The authors are grateful to Dr. D. Yu. Sorokin (Vinogradsky Institute of Microbiology, Russian Academy of Sciences) for providing the culture of *Tv. nitratireducens* strain ALEN 2 and for participating in the discussion of the paper and to Dr. K. B. Shumaev (Bach Institute of Biochemistry, Russian Academy of Sciences) for his help in EPR experiments.

This work was supported by the Russian Foundation for Basic Research (grant 07-04-01559a).

## REFERENCES

1. Jepson, B. J., Mohan, S., Clarke, T. A., Gates, A. J., Cole, J. A., Butler, C. S., Butt, J. N., Hemmings, A. M., and Richardson, D. J. (2007) *J. Biol. Chem.*, **282**, 6425-6437.
2. Zumft, W. G. (1997) *Microbiol. Mol. Biol. Rev.*, **61**, 533-616.
3. Stolz, J. F., and Basu, P. (2002) *Chem. Bio. Chem.*, **3**, 198-206.
4. Richardson, D. J., Berks, B. C., Russell, D. A., Spiro, S., and Taylor, C. J. (2001) *Cell. Mol. Life Sci.*, **58**, 165-178.
5. Philippot, L., and Hojberg, O. (1999) *Biochim. Biophys. Acta*, **1446**, 1-23.
6. Jepson, B. J., Anderson, L. J., Rubio, L. M., Taylor, C. J., Butler, C. S., Flores, E., Herrero, A., Butt, J. N., and Richardson, D. J. (2004) *J. Biol. Chem.*, **279**, 32212-32218.
7. Martinez-Espinosa, R. M., Marhuenda-Egea, F. C., and Bonete, M. J. (2001) *FEMS Microbiol. Lett.*, **204**, 381-385.
8. Lin, J. T., and Stewart, V. (1998) *Adv. Microb. Physiol.*, **39**, 1-30.
9. Bender, R. A., and Friedrich, B. (1990) *J. Bacteriol.*, **172**, 7256-7259.
10. Moreno-Vivian, C., Cabello, P., Martinez-Luque, M., Blasco, R., and Castillo, F. (1999) *J. Bacteriol.*, **181**, 6573-6584.
11. Berks, B. C., Ferguson, S. J., Moir, J. W., and Richardson, D. J. (1995) *Biochim. Biophys. Acta*, **1232**, 97-173.
12. Reyes, F., Roldan, M. D., Klipp, W., Castillo, F., and Moreno-Vivian, C. (1996) *Mol. Microbiol.*, **19**, 1307-1318.
13. Sorokin, D. Y., Tourova, T. P., Sjollema, K. A., and Kuenen, J. G. (2003) *Int. J. Syst. Evol. Microbiol.*, **53**, 1779-1783.
14. Tikhonova, T. V., Slutsky, A., Antipov, A. N., Boyko, K. M., Polyakov, K. M., Sorokin, D. Y., Zvyagilskaya, R. A., and Popov, V. O. (2006) *Biochim. Biophys. Acta*, **1764**, 715-723.
15. Tikhonova, T. V., Slutskaya, E. S., Filimonenkov, A. A., Boyko, K. M., Kleimenov, S. Y., Konarev, P. V., Polyakov, K. M., Svergun, D. I., Trofimov, A. A., Khomenkov, V. G., Zvyagilskaya, R. A., and Popov, V. O. (2008) *Biochemistry (Moscow)*, **73**, 164-170.
16. Pfenning, N., and Lippert, K. (1966) *Arch. Microbiol.*, **55**, 245-256.
17. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
18. Nesterenko, M. V., Tilley, M., and Upton S. J. (1994) *J. Biochem. Biophys. Meth.*, **28**, 239-242.
19. Nicolas, D. J. D., and Nason, A. (1957) *Meth. Enzymol.*, **3**, 981-954.

20. Devis, B. J., and Ann, N. Y. (1964) *Acfd. Sci.*, **121**, 404-407.
21. Sakurai, N., Asada, A., Mano, S., Kataoka, K., and Sakurai, T. (2006) *DNA Seq.*, **17**, 363-369.
22. Hettmann, T., Anemuller, S., Borcherding, H., Mathe, L., Steinrucke, P., and Diekmann, S. (2003) *FEBS Lett.*, **534**, 143-150.
23. Yoshimatsu, K., Sakurai, T., and Fujiwara, T. (2000) *FEBS Lett.*, **470**, 216-220.
24. Ballard, A. L., and Ferguson, S. J. (1988) *Eur. J. Biochem.*, **174**, 207-212.
25. Moore, R. W., Welton, A. F., and Aust, S. D. (1978) *Meth. Enzymol.*, **52**, 324-331.
26. Blasco, F., Guigliarelli, B., Magalon, A., Asso, M., Giordano, G., and Rothery, R. A. (2001) *Cell. Mol. Life Sci.*, **58**, 179-193.
27. Bertero, M. G., Rothery, R. A., Palak, M., Hou, C., Lim, D., Blasco, F., Weiner, J. H., and Strynadka, N. C. (2003) *Nat. Struct. Biol.*, **10**, 681-687.
28. Jormakka, M., Richardson, D., Byrne, B., and Iwata, S. (2004) *Structure*, **12**, 95-104.
29. Rothery, R. A., Bertero, M. G., Cammack, R., Palak, M., Blasco, F., Strynadka, N. C. J., and Weiner, J. H. (2004) *Biochemistry*, **43**, 5324-5333.
30. Hochstein, L. I., and Tomlinson, G. A. (1988) *Annu. Rev. Microbiol.*, **42**, 231-261.
31. Bell, L. C., Richardson, D. J., and Ferguson, S. J. (1990) *FEBS Lett.*, **265**, 85-87.
32. Antipov, A. N., Morozkina, E. V., Sorokin, D. Y., Golubeva, L. I., Zvyagilskaya, R. A., and L'vov, N. P. (2005) *Biochemistry (Moscow)*, **70**, 799-803.