Molybdenum-free nitrate reductases from vanadate-reducing bacteria

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Abstract Two catalytically distinct molybdenum-free dissimilatory nitrate reductases, a soluble periplasmic and a membrane-bound one, were isolated from the vanadate-reducing facultatively anaerobic bacterium *Pseudomonas isachenkovii* and purified to electrophoretic homogeneity. The enzymes did not contain molybdenum, the periplasmic enzyme contained vanadium, whereas the membrane-bound enzyme was vanadium-free. Both nitrate reductases lacked molybdenum cofactor. This fact was proved by reconstitution of the apoprotein of the nitrate reductase of *Neurospora crassa nit-1* mutant. This is the first demonstration of molybdenum-free and molybdenum cofactor-free nitrate reductases.

Key words: Nitrate reductase; Vanadium-containing enzyme; Denitrification

1. Introduction

Nitrate reductase is a key enzyme of eukaryotic nitrate assimilation and prokaryotic nitrate assimilation and dissimilation. The requirement for molybdenum in nitrate reduction was first reported by Wolfe [1]. Molybdenum is incorporated in the active site of nitrate reductase as molybdocofactor (Moco) [2]. All nitrate reductases that have so far been purified to homogeneity were shown to contain molybdenum. Moreover, vanadium (instead of molybdenum) was not incorporated either into conventional molybdenum-containing nitrate reductases from plants [3] and fungi [4] or into Moco-containing protein (free of molybdenum) from pea seeds [5]. We suggested that vanadate-reducing bacteria isolated from vanadium-enriched habitats [6,7] could serve as a promising source for isolation of vanadium-containing nitrate reductases.

The function of vanadium in cellular metabolism is poorly understood. Subtoxic doses of vanadium influence sulfur metabolism, decrease the rate of respiration and oxidative phosphorylation, and inhibit many enzymes [8]. The involvement of vanadium instead of molybdenum in alternative nitrogenases was first demonstrated in *Azotobacter* strains with deleted structural genes for conventional molybdenum-containing nitrogenases [9]. Evidence for the existence of molybdenum-free nitrogenases in a variety of organisms has been reported [10]. After the description of the vanadium-containing nitrogenase, a second alternative nitrogenase apparently lacking any metals other than iron was isolated [11]. Only the data were reported by Dr. Singh's group [12,13] that vanadium, but not molybdenum, stimulates nitrate assimilation by a tungsten-resistant mutant of *Nostoc muscorum*.

Here we describe, for the first time, two nitrate reductases of a novel type lacking molybdenum and Moco, one of them containing vanadium.

2. Materials and methods

2.1. The organism and growth conditions

In this work, we used the facultatively anaerobic and facultatively chemolithotrophic vanadate-reducing bacterium *Pseudomonas isachenkovii* isolated from an ascidium worm [6] containing 18% vanadium in the blood chromogen [14]. This bacterium can use a number of organic compounds as electron donors and molecular hydrogen and carbon monoxide for the reduction of oxygen, vanadate and nitrate. During dissimilation, pentavalent vanadium is reduced to the tetra- and trivalent states and nitrate to molecular nitrogen. The bacterium is highly resistant to toxic pentavalent vanadium and is able to grow in the presence of 5 g of vanadium per liter. Although the standard redox potential of vanadate reduction is higher than that of nitrate reduction, calculations [15] showed that in the simultaneous presence of nitrate and vanadate in the medium, vanadate reduction should follow the complete reduction of nitrate.

Cells were grown anaerobically at 29–30°C in 10-l bottles in modified Postgate medium B containing (g/l): KH_2PO_4, 0.25; NH_4Cl, 0.5; KNO_3, 1.0; $NaVO_3{\cdot}2H_2O$, 0.5; sodium lactate, 1.0 and yeast extract, 0.2. The basal medium was also supplemented with microelements and traces of $MgSO_4{\cdot}7H_2O$ and $FeSO_4{\cdot}7H_2O$.

The cells were harvested when the medium turned a blue color due to tetravalent vanadium formation, which indicated nitrate depletion. The cells were collected by centrifugation, passed through a French press at 8000 psi and the membranes were separated by centrifugation at $150\,000\times g$ for 60 min. The supernatant was used for periplasmic nitrate reductase purification. To solubilize the membrane-bound enzyme, isolated membranes were incubated with 6% (v/v) Triton X-100 in 50 mM phosphate buffer, pH 7.0 at 4°C for 12 h. The resulting crude extract was clarified by centrifugation at $15\,000\times g$ for 20 min. The periplasmic and solubilized membrane fractions were kept in liquid nitrogen until use.

2.2. Purification of nitrate reductases

The enzymes were purified to electrophoretic homogeneity using a combination of gel filtration on Toyopearl HW-55, chromatography on DEAE-Toyopearl 650M, absorbtion chromatography on hydroxyapatite (HTP-Bio Gel) and hydrophobic chromatography on octyl-Sepharose.

Active subunits of nitrate reductases were obtained by treating the oligomeric enzymes with 1 M KCl in 0.25 mM K-phosphate buffer, pH 7.0, for 20 min, followed by separation on octyl-Sepharose (for the membrane-bound enzyme).

2.3. Assay of nitrate reductase activity

The assay of nitrate reductase was carried out at 70°C using reduced forms of methyl viologen as reductant as described in [17]. Specific activity of nitrate reductase was expressed as μmol NO_2 formed per min per mg of protein. Nitrate reductase activity in the gel was detected as described [18] at 70°C.

2.4. Reconstitution of molybdenum cofactor with extract of *Neurospora crassa nit-1* mutant

The assay of the Moco involved the reconstitution of the mono-

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meric apo subunits of the nitrate reductase to yield an active dimer in extracts of nitrate-induced mycelia of *N. crassa nit-1* mutant. The growth conditions for the *nit-1* mutant and preparation of cell-free extract were described earlier [16]. The assay of Moco was performed essentially as described previously [19]. The aliquots of homogeneous preparations of nitrate reductases (200 μg of protein) were heat-treated (90°C, 1 min) or acid-treated (up to a final pH value of 2.5), with reduced glutathione or ascorbic acid (final concentration 2 mM) as protectors of Moco. The treated enzymes were incubated with 100 μl of crude extract of *nit-1* mutant in the presence of 2 mM sodium molybdate or sodium vanadate at 5°C for 24 h [19]. Buttermilk xanthine oxidase was used as a reference.

To detect the presence of molybdopterin in enzyme preparations, fluorescence emission spectra were recorded on an MPF-4 Hitachi fluorescence spectrometer with an excitation wavelength of 360 nm (λ emission at 450 nm) after complete oxidation by autoclaving of samples [20].

2.5. Gel electrophoresis

The molecular mass (M_r) of enzyme subunits was determined by SDS-PAGE according to [21] using 10% acrylamide gel in 370 mM Tris-HCl buffer (pH 8.8) for the separation gel and 2.5% acrylamide in 58 mM Tris-HCl buffer (pH 6.9) for the stacking gel. β-Galactosidase (116 kDa), phosphorylase b (97,4), bovine serum albumin (67), ovalbumin (45), and carbonic anhydrase (29) were chosen as standards. The native M_r of nitrate reductase was determined using gel electrophoresis which was carried out in 5–15% (w/v) gradient polyacrylamide gels in Tris-HCl buffer pH 8.8 by the method of Davis [22]. M_r markers were urease (240 kDa), aldolase (147), bovine serum albumin (67), ovalbumin (45), and carbonic anhydrase (29). Proteins were silver stained according to [23].

Polyacrylamide gel isoelectric focusing was performed using a 5% gel containing 2% (w/v) ampholine (pH 4.0–9.0). Isoelectric focusing was carried out at 10 W constant power and 4°C for 6 h. The p*I* of the enzymes was estimated by comparison with the migration of standard proteins.

2.6. Metal determination

The metal content in the periplasmic nitrate reductase was assayed by X-ray microanalysis and by laser mass spectrometry. X-ray microanalysis was carried out on a JEM-100C XII electron microscope equipped with an EM-ASID4D scanning unit and a Link 860 X-ray analyzer with an E 5123 detector.

Determination of metals by laser mass spectrometry was performed using a Lasma mass spectrometer, constructed by the Space Research Institute of the Russian Academy of Sciences and described in [24].

Vanadium was estimated by catalytic method with gallic acid [25] after wet combustion of enzyme preparations with a mixture of HNO_3:$HClO_4$ (2:1) and separation from iron by adding ammonium fluoride.

2.7. Protein determination

Protein was estimated by the Bradford procedure [26] using crystalline bovine serum albumin as a standard.

3. Results and discussion

Two types of dissimilatory nitrate reductases (membrane-bound and soluble periplasmic) from anaerobically grown vanadate-reducing *P. isachenkovii* were purified to homogeneity (Fig. 1). The membrane-bound enzyme (330 kDa) (Fig. 1A, lanes 3 and 5) was composed of two non-identical subunits with molecular masses of 130 and 67 kDa, respectively (Fig. 1B, lane 1). Molecular organization of this type is characteristic for the most dissimilatory membrane-bound nitrate reductases isolated from microorganisms [27]. The active periplasmic enzyme (220 kDa) (Fig. 1A, lanes 4 and 6) was composed of subunits with a molecular mass of approximately 55 kDa (Fig. 1B, lane 2). The large subunit of the membrane-bound enzyme (130 kDa) and the monomeric form of the periplasmic enzyme (55 kDa) possessed their own nitrate reductase activity (Fig. 1A, lanes 1 and 2). This is the first demonstration of nitrate reductase activity of individual subunits of the enzyme.

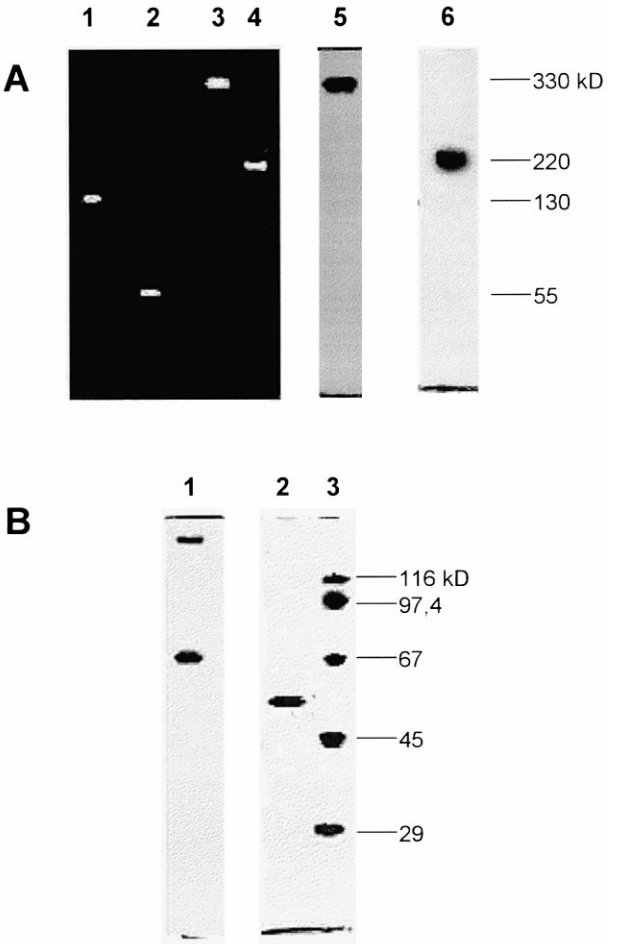

Fig. 1. Polyacrylamide gel electrophoresis of purified membrane-bound and periplasmic nitrate reductases from *P. isachenkovii*. A: Non-denaturating gradient gel (5–15%) electrophoresis. Lanes 1–4: identification of nitrate reductase activities of subunits (1, membrane-bound and 2, periplasmic enzymes) and native enzymes (3, membrane-bound and 4, periplasmic enzymes). Lanes 5 and 6: protein stained with silver stain (membrane-bound and periplasmic enzyme), respectively. B: SDS-PAGE. Lanes 1 and 2: membrane-bound and periplasmic enzyme, respectively, stained with silver stain. Lane 3: standard protein markers.

Specific activities of the homogeneous membrane-bound and periplasmic nitrate reductases with 10 μM nitrate as a substrate were 60–70 and 20–30 μmol NO_2 min^{-1} mg^{-1} of protein, respectively, which is comparable with that of dissimilatory nitrate reductases isolated previously [27].

Determination of the metal contents in the periplasmic enzyme (Fig. 2A,B) showed the absence of molybdenum and the presence of vanadium. There is a correlation between vanadium content and nitrate reductase activity during the course of elution of the periplasmic enzyme from the column with HTP-Bio Gel (Fig. 2C).

Using the same methods for the membrane-bound nitrate reductase (data not shown) we failed to find any metals except chromium, probably as a contamination.

The properties of two isolated nitrate reductases are summarized in Table 1.

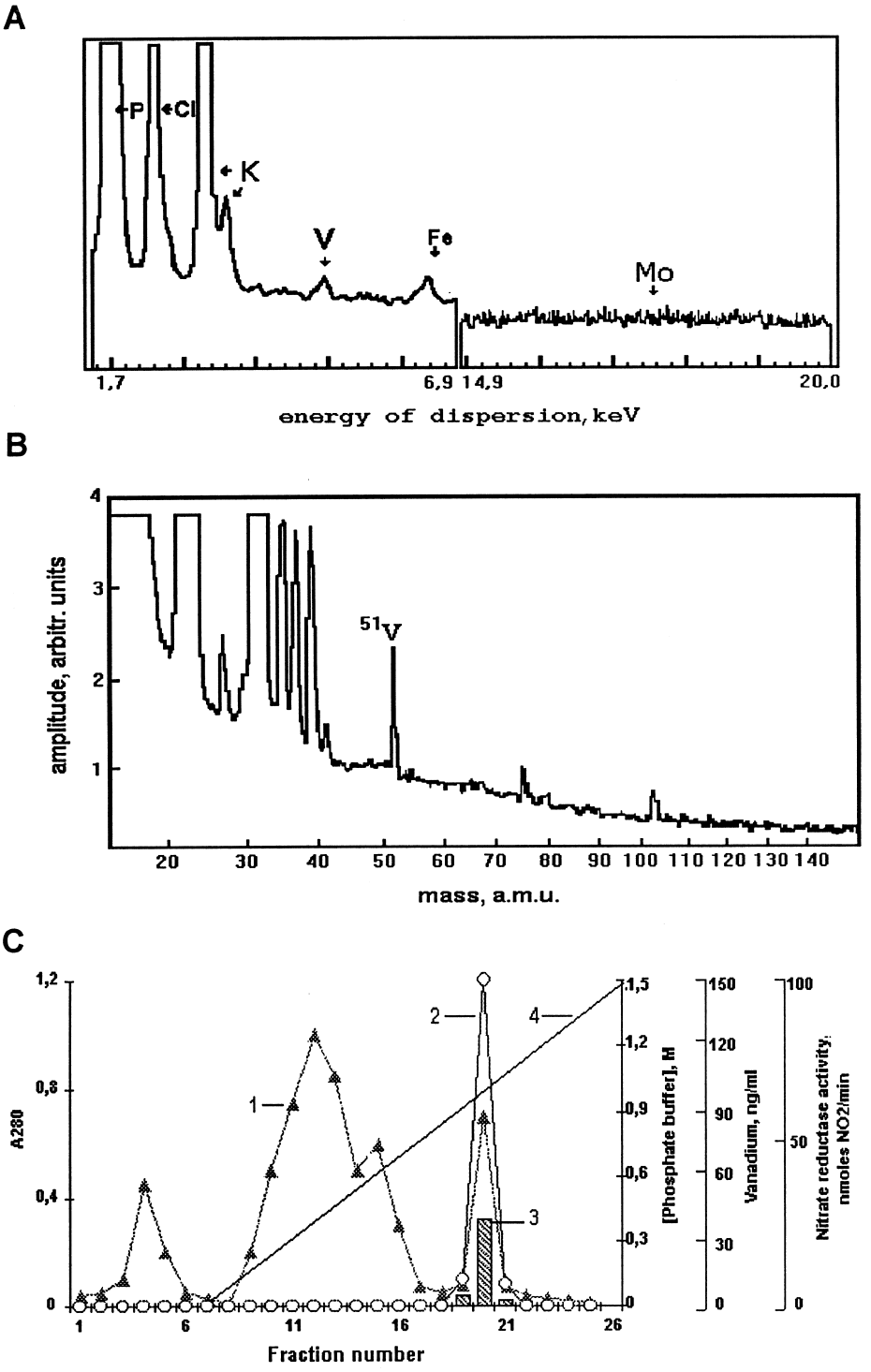

Fig. 2. Metal content in purified periplasmic nitrate reductase from *P. isachenkovii*. A: X-ray microanalysis. B: Laser mass spectrometry. C: Correlation of vanadium content with protein and nitrate reductase activity. The figure shows the elution profile of the periplasmic nitrate reductase from the column of HTP-Bio gel. 1: eluted protein (A 280); 2: nitrate reductase activity; 3: vanadium content; 4: phosphate buffer.

Reduced methyl viologen was the most effective electron donor for both the membrane-bound and the periplasmic nitrate reductases.

The optimum pH was found to be 6.8, but fairly good activity could be measured over the pH range 6–9. An exceptionally high temperature optimum at 70–80°C, unusual for enzymes isolated from mesophilic microorganisms, was found.

It is well known that all molybdenum-containing nitrate reductases isolated so far contained Moco as an active site of the enzyme [2]. However, all our attempts to detect Moco and molybdopterin in the purified nitrate reductases were unsuccessful. It is reasonable to suggest that vanadate-reducing bacteria realize a Moco-independent pathway for nitrate dissimilation.

Table 1
Properties of two molybdenum-free nitrate reductases from the vanadate-reducing bacterium *P. isachenkovii*

Intracellular location	Membrane-bound	Periplasmic
Metal content	chromium[a]	vanadium
Electron donor	reduced methyl viologen	reduced methyl viologen
M_r of native enzyme (kDa)	330	220
Subunit composition (kDa)	130; 67	55
Catalytically active subunit (kDa)	130	55
Optimum pH	6.0–8.5	6.0–8.5
Optimum temperature (°C)	70	80
Isoelectric point (p*I*)	4.0	4.0
Inhibitors: ($I_{0.5}$ (M))		
NaN_3	60	20
KCN	70	40
Moco and molybdopterin fluorescence[b]	not detected	not detected

[a]Possibly as a contamination.
[b]Assay for the molybdenum cofactor was performed with of *N. crassa nit-1* mutant.

Like conventional molybdenum-containing dissimilatory nitrate reductases, the enzymes from *P. isachenkovii* were inhibited by low concentrations of cyanide, an inhibitor of the respiratory chain, and azide, a metal-chelating agent (Table 1).

The results obtained show that *P. isachenkovii* can express two catalytically distinct molybdenum-free and molybdenum cofactor-free dissimilatory nitrate reductases, one of which contains vanadium.

We would like to stress that the enzymes isolated differ significantly from all known nitrate reductases described so far. They can probably be regarded as belonging to a novel type of dissimilatory nitrate reductases.

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