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SHORT COMMUNICATION

Isolation and preliminary characterization of a respiratory nitrate reductase from hydrocarbon-degrading bacterium *Gordonia alkanivorans* S7

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Abstract *Gordonia alkanivorans* S7 is an efficient degrader of fuel oil hydrocarbons that can simultaneously utilize oxygen and nitrate as electron acceptors. The respiratory nitrate reductase (Nar) from this organism has been isolated using ion exchange chromatography and gel filtration, and then preliminarily characterized. PAGE, SDS-PAGE and gel filtration chromatography revealed that Nar consisted of three subunits of 103, 53 and 25 kDa. The enzyme was optimally active at pH 7.9 and 40°C. K_m values for NO_3^- (110 μM) and for ClO_3^- (138 μM) were determined for a reduced viologen as an electron donor. The purified Nar did not use NADH as the electron donor to reduce nitrate or chlorate. Azide was a strong inhibitor of its activity. Our results imply that enzyme isolated from *G. alkanivorans* S7 is a respiratory membrane-bound nitrate reductase. This is the first report of purification of a nitrate reductase from *Gordonia* species.

Keywords Respiratory nitrate reductase · Purification · *Gordonia alkanivorans*

Abbreviations

Nar	Respiratory nitrate reductase
Nas	Assimilatory nitrate reductase
DTT	Dithiothreitol
MV	Methyl viologen
EDTA	Ethylenediaminetetraacetic acid
pCMB	*p*-Chloromercuribenzoate

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Introduction

Bacteria can express multiple, genetically and biochemically distinct nitrate reductases. Three classes of these enzymes can be identified in prokaryotes: a class of assimilatory, cytoplasmic nitrate reductases (Nas) and two classes of dissimilatory reductases such as respiratory, membrane-bound nitrate reductases (Nar) and periplasmic nitrate reductases (Nap). The first of dissimilatory enzymes (Nar) plays a key role in the generation of metabolic energy by using nitrate as a terminal electron acceptor (nitrate respiration), and it couples the reduction of nitrate to the generation of ATP in the absence or at low concentrations of oxygen. The second of them (Nap) participates in the dissipation of excessive reducing power for redox balancing (nitrate dissimilation). These enzymes differ in their cellular location, function, subunit composition and the number and identity of their redox centers [1–5]. Dissimilatory nitrate reductases differ also in structure, gene sequence and biochemical properties. Membrane-bound respiratory nitrate reductases can use NADH as an electron donor and reduce chlorate. They are strongly inhibited by low concentrations of azide. Periplasmic nitrate reductases are insensitive to low azide concentration and neither reduce chlorate nor use NADH as an electron donor. Therefore, these two enzymatic activities can be distinguished based on their properties [6]. Bacterial respiratory membrane-bound nitrate reductases are integral protein complexes with the active site located on the cytoplasmic

face of the cytoplasmic membrane. Some Nar enzymes are composed of three subunits: a catalytic α subunit (NarG), a soluble β subunit (NarH) and γ subunit (NarI). Two subunits, α and β, constitute the cytoplasmic domain, whereas the γ subunit constitutes the membrane domain that binds α and β subunits to the cytoplasmic side of the inner membrane and accepts electrons from the quinol pool and transfers them via its *b*-haems to the β subunit. Nar can be extracted from membranes with detergents in an intact form composed of these three subunits [4, 5, 7, 8]. Only several membrane-bound respiratory reductases from *Escherichia coli*, *Aerobacter aerogenes*, *Haloarcula marismortui*, *Haloferax mediterranei*, *Paracoccus denitrificans*, *Pseudomonas chlororaphis* and *Pseudomonas aeruginosa* have been isolated and characterized up to now [4, 7, 9–15].

Gordonia species have attracted much interest in recent years since they are capable of degrading xenobiotics, environmental pollutants or other slowly biodegradable natural polymers [16]. A denitrifying bacterial strain *G. alkanivorans* S7 was found to be an efficient degrader of fuel oil hydrocarbons. This work focused on isolation, purification and preliminary characterization of the respiratory nitrate reductase (Nar) from this strain.

Materials and methods

Microorganism

An aerobic, gram-positive, hydrocarbon-degrading strain, *G. alkanivorans* S7, from the collection of the Institute of Technical Biochemistry, Technical University of Lodz, was maintained at 4°C on Luria-Bertani (LB) agar coated with a film of diesel oil hydrocarbons.

Culture conditions and preparation of crude cell-free extracts

The strain was cultured in a liquid medium containing (grams per liter of tap water): glucose, 2.0; yeast extract, 2.0; Na_2HPO_4, 1.5; $NaNO_3$, 4.0; 3.75% (v/v) diesel oil as carbon source. The pH of the culture medium was adjusted to 6.5. *G. alkanivorans* S7 was grown for 5 days at 30°C under relatively oxygen-limiting conditions due to the presence of a hydrophobic substrate (in 500-ml spherical flasks containing 80 ml of culture medium) on a rotary shaker at 200 rpm. Cells were harvested by centrifugation (18,000×*g*, 20 min, 4°C) and washed with 10 mM Tris-HCl buffer pH 7.6 (5× volume of the cell pellet). The pellet was resuspended in the same buffer and disrupted by sonification (8 × 3 min, 4°C, 150 W). The supernatant from centrifuging (106,000×*g*, 60 min, 4°C) containing the cytoplasmic fraction was discarded. The pellet (membrane fraction) was resuspended in 10 mM Tris-HCl buffer pH 7.6 supplemented with Triton X-100 (20% w/v). This suspension was gently stirred overnight and then centrifuged at 30,000×*g* for 30 min at 4°C. The supernatant was dialyzed against 50 mM phosphate buffer pH 7.3 and subjected to two-step column chromatography yielding the purified respiratory nitrate reductase.

Chemicals

Yeast extract was purchased from Difco, USA, and diesel oil was obtained from "PKN ORLEN," Poland. All other chemicals were purchased from Sigma-Aldrich, USA.

Purification of respiratory nitrate reductase

The dialyzed supernatant (31.5 ml) obtained as above was applied on a DEAE Biogel A column (2.6 × 30 cm), previously equilibrated with 50 mM phosphate buffer pH 7.3. Elution was carried out with an increasing linear gradient of 0.2–0.5 M NaCl in the same buffer at a flow rate of 32 ml/h. Fractions containing Nar were loaded on a Sephacryl S-300 HR column (0.8 × 27.5 cm), previously equilibrated with 50 mM phosphate buffer pH 7.3. The column was washed with the same buffer with a flow rate of 20 ml/h.

Protein and nitrate reductase assays

The protein content was determined by Bradford method [17], with bovine serum albumin as a standard or spectrophotometrically at 280 nm. Nar activity, measured as the rate of nitrate to nitrite conversion, was estimated by colorimetric determination of nitrite at 520 nm using dithionite reduced methyl viologen as the electron donor. The assay mixture contained (total volume of 1.1 ml) 100 mM Tris-HCl buffer (pH 8.0), 4 mM methyl viologen, 35 mM KNO_3, 17 mM $Na_2S_2O_4$ (freshly prepared) in 0.1 M $NaHCO_3$ and enzyme preparation. The assay was carried out at 40°C for 5 min. The nitrite content was determined by the diazo-coupling procedure [18]. The unit of nitrate reductase activity is expressed as micromoles of NO_2^- appearing in 1 min.

Biochemical characterization

Molecular mass of Nar was determined by electrophoresis under non-denaturing conditions in 8 and 10% polyacrylamide gels at pH 8.3 as described by Davis [19] with an Amersham high molecular weight calibration kit for native electrophoresis and by SDS-PAGE according to Laemmli [20] with an Amersham low molecular weight calibration

kit for SDS electrophoresis. The molecular mass of Nar was also determined by gel filtration using a Sepharose 6B column (1.2 × 88 cm) equilibrated with 50 mM Tris-HCl buffer pH 7.0 and calibrated with Sigma standard proteins of known Mr. The optimum pH for Nar activity was determined with methyl viologen as the electron donor and nitrate in Tris-HCl buffer at pH 6.85–10.35 and 40°C. Enzyme activity was also assayed at 25–70°C and pH 7.90. The influence of various substances on Nar activity was determined for their ultimate concentration of 1 mM.

Kinetic parameters of nitrate reductase from *G. alkanivorans* S7 were determined using different concentrations of nitrate or chlorate (0.5–2.5 mM) and methyl viologen as the electron donor (4 mM).

Results are presented as the mean (± standard deviation) of triplicate assays. Statistical analysis of results was based on Student's *t* test ($P = 99\%$).

Results and discussion

Purification of nitrate reductase

The dialyzed supernatant (obtained as described in "Materials and methods") was subjected to ion exchange chromatography on DEAE Biogel A. Only the second of two protein peaks contained Nar activity (Fig. 1). It was eluted with the NaCl gradient in the range of 0.38–0.42 M. Active fractions were pooled and applied to Sephacryl S-300 HR. The nitrate reductase was eluted as a single peak (Fig. 2). The enzyme was purified 149-fold (as compared to the crude cell-free extract) with a yield of *circa* 13% (Table 1). Separation of Nar from other membrane proteins was successful, although this process is generally difficult because membrane proteins tend to aggregate with each other and with Triton X-100 molecules [7]. The specific activity of Nar was 0.270 U/mg of protein. Published specific activities of respiratory nitrate reductases span 4–5 orders of magnitude, from 350 U/mg for enzyme from *Pyrobaculum aerophilum* to 0.23 U/mg for *Rhodobacter capsulatum* reductase [21]. Because conditions and procedures of these assays were different, we think that the discrepancies between the specific activity values do not reflect the true differences between known respiratory nitrate reductases.

Biochemical characterization

Gel filtration chromatography on Sepharose CL 6-B revealed that molecular mass of Nar was close to 443 kDa. Native gel electrophoresis of isolated reductase showed a single protein band (when stained with silver nitrate) corresponding to the similar molecular mass of 435 kDa (Fig. 3).

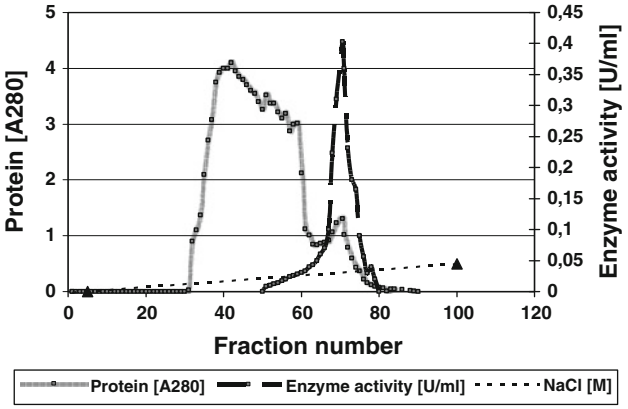

Fig. 1 Elution profile of nitrate reductase activity from DEAE-Biogel A column

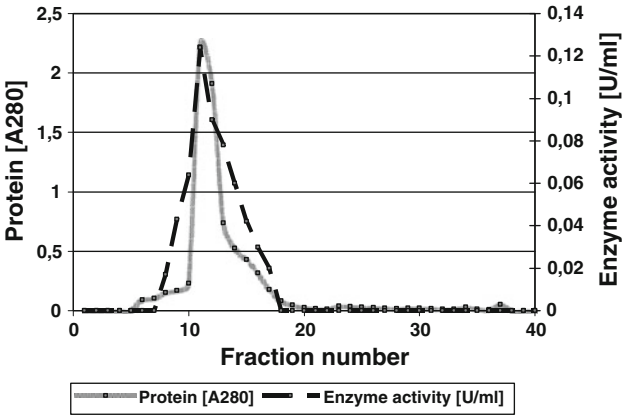

Fig. 2 Elution profile of nitrate reductase activity from Sephacryl S-300 HR column

Although these results are consistent, they can result from aggregation of molecules of *G. alkanivorans* S7 nitrate reductase. The tendency to aggregation was found to change with the method of enzyme extraction from membrane [22]. This phenomenon is characteristic of hydrophobic proteins and was also observed for nitrate reductases from *A. aerogenes* [9] and from *E. coli* K12 [11]. Molecular mass of the first enzyme estimated by gel filtration was close to 400 kDa. M_r of the second enzyme determined on agarose column and by ultracentrifugation was 720 and 773.6 kDa, respectively, while SDS-PAGE revealed that the enzyme is composed of two different subunits of 142 and 58 kDa. Some authors assumed that M_r of this nitrate reductase was the sum of mass of its two subunits [13, 21]. SDS-PAGE of enzyme isolated from *G. alkanivorans* S7 revealed three protein bands (when stained with silver nitrate) of M_r 103, 53 and 25 kDa (Fig. 4). They were designated α, β and γ by analogy with the enzyme from *E. coli*. Migration of isolated membrane-bound nitrate reductase on SFS-PAGE did not correlate with formula molecular weights, and "gel shifting" effect

Table 1 Purification of respiratory nitrate reductase from *G. alkalivorans* S7

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	31.5	968.60	2.131	0.0022	1.0	100
DEAE Biogel A	39.5	32.50	2.013	0.0620	28.2	94.5
Sephacryl S-300 HR	7.5	0.83	0.270	0.3270	148.6	12.6

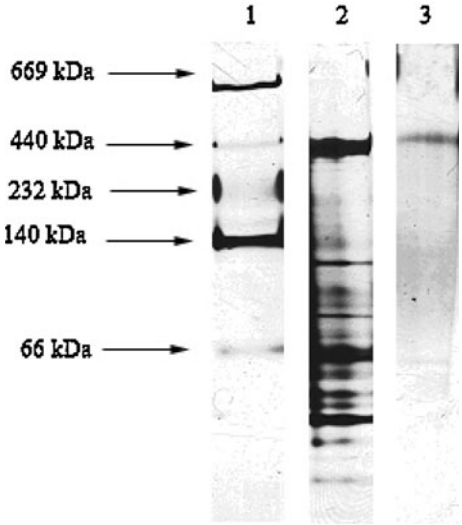

Fig. 3 PAGE of nitrate reductase from *G.alkanivorans* S7: *lane 1* standard proteins with known molecular masses; *lane 2* fractions from DEAE Biogel A; *lane 3* fractions from Sephacryl S-300 HR

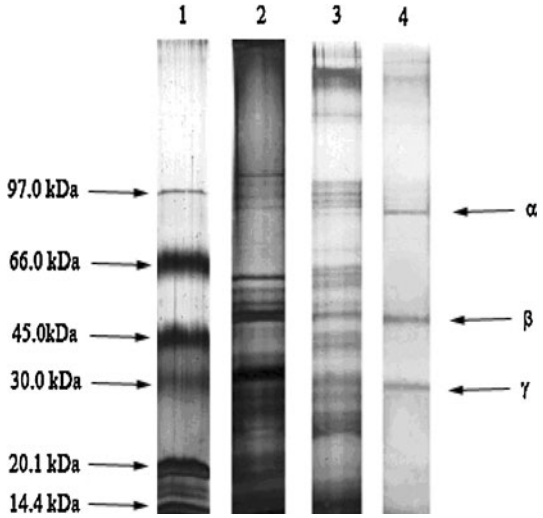

Fig. 4 SDS-PAGE of nitrate reductase from *G.alkanivorans* S7: *lane 1* standard proteins with known molecular masses; *lane 2* crude extract; *lane 3* fractions from DEAE Biogel A; *lane 4* fractions from Sephacryl S-300 HR

was observed, which appears to be common for membrane proteins [23]. Also some other membrane-bound nitrate reductases consist of three subunits [7]. Their number depends also on the method of enzyme isolation.

The optimum pH for activity of nitrate reductase from *G. alkanivorans* S7 was approximately 7.90. It was optimally active at 40°C. Optimum pH and temperature conditions for activity of other known nitrate reductases depend on their producers and growth conditions. For instance, Nar from *P. aerophilum* was optimally active at pH 6.5 and 95°C [21], while the optimum temperature for activity of enzyme isolated from halophilic strain *H. mediterranei* was close to 70°C. Surprisingly, the latter was optimally active at two different pHs (7.9 and 8.2) when nitrate reduction was carried out at 40 and 60°C, respectively [4].

The reduced methyl viologen (MV) was the best electron donor for nitrate (0.327 U/mg) and chlorate reduction (0.229 U/mg) for Nar from *G. alkanivorans* S7 (Table 2). This respiratory nitrate reductase used also the reduced benzyl viologen (0.312 U/mg), but it did not use NADH as the electron donor in contrast to the other Nar enzymes but like nitrate reductase isolated from *H. mediterranei* [4, 6].

Table 2 Substrate specifity of respiratory nitrate reductase from *G. alkalivorans* S7

Substrate 35 mM	Electron donor	Relative activity (%)
KNO_3	Methyl viologen	100.0
$KClO_3$		70.0
KNO_3	Benzyl viologen	95.5
	NADH	0

Inhibition studies revealed that neither Na_2SO_3 nor EDTA was an effective inhibitor (a decrease in activity was close to 70.0 and 62.0%, respectively), whereas dithiothreitol and pCMB were more potent inhibitors (a decrease in activity of 56 and 52%, respectively) of *G. alkanivorans* S7 nitrate reductase. Azide, which inhibits enzymes by metal chelation, acted as the potent inhibitor (a drop in activity to 44%) thereby providing evidence that Nar from *G. alkanivorans* S7 ranked among respiratory nitrate reductases. Azide was also the potent inhibitor of enzymes isolated from *Bradyrhizobium japonicum*, *P. aerophilum*, *P. denitryficans* and *H. mediterranei* [14, 24, 25].

K_m values for NO_3^- (110 μM) and for ClO_3^- (138 μM) were determined for a reduced viologen as an electron donor. Also the majority of nitrate reductases described in the literature prefer nitrate to chlorate [22]. The apparent K_m of Nar from *G. alkanivorans* S7 for nitrate was close to values reported for the other nitrate reductases [4]. For instance, the K_m values for enzyme isolated from *P. denitrificans* for NO_3^- and ClO_3^- were 285 and 470 μM, respectively [14]. The relatively high affinity for nitrate (K_m of 20 μM) was reported for *Desulfovibrio desulfuricans* ATCC 27774 nitrate reductase, whereas K_m for *E. coli* enzyme was more than 70-fold higher (1,500 μM, the lowest reported affinity) [11, 26].

Our results imply that the nitrate reductase purified from *G. alkanivorans* S7 is a respiratory membrane nitrate reductase (Nar). To elucidate the correlation among the properties, structure and function of enzyme isolated from *G. alkanivorans* S7, further physicochemical and genetic investigations are necessary.

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