Acta Biochimica Polonica

Vol. 55 No. 4/2008, 753–760

on-line at: www.actabp.pl

Regular paper

Mass spectrometry identification of membrane-bound respiratory nitrate reductase from *Bradyrhizobium* sp. (*Lupinus*)*

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Received: 10 July, 2008; revised: 24 September, 2008; accepted: 25 October, 2008
available on-line: 17 November, 2008

Respiratory nitrate reductase (NR) from *Bradyrhizobium* sp. (*Lupinus*) USDA 3045 has biochemical properties of the membrane-bound NR type. However, in the completely sequenced rhizobium genomes only genes for the periplasmic type of dissimilatory NR were found. Therefore purification and identification of the enzyme by tandem mass spectrometry (MS/MS) was undertaken. MS/MS spectra representing 149 unique tryptic peptides derived from purified 137-kDa subunit matched the NCBInr-deposited NarG sequences. MS/MS sequencing of two other SDS/PAGE bands (65- and 59-kDa) identified them as derivatives of the NarH-type protein. Applying additional validation criteria, 73% of the sequence of the NarG subunit (902 aa) and 52% of NarH sequence (266 aa) was assembled (UniProt KB acc. no. P85097 and P85098). This is the first unambiguous identification of an active NarGH-like NR in rhizobia. Moreover, arguments are provided here for the existence of a functional enzyme of this type also among other rhizobial species, basing on immunoblot screening and the presence of membrane-associated NR-active electrophoretic forms.

Keywords: *Bradyrhizobium* sp. (*Lupinus*), enzyme purification, immunoblot screening, mass spectrometry sequencing, membrane-bound nitrate reductase [EC 1.7.99.4], rhizobia

INTRODUCTION

Bradyrhizobium sp. (*Lupinus*) USDA 3045 strain is able to live in symbiosis with two green manure crops: *Ornithopus sativus* and *Lupinus luteus*. Our previous work showed that anaerobically grown free-living cells of this strain had a nitrate reductase (NR) activity, efficiently linked with energy conservation (Polcyn & Luciński, 2003), being therefore considered as respiratory (Moreno-Vivián & Ferguson, 1998). To date, two general types of dissimilatory NRs have been recognized in bacterial cells: membrane-bound NarGHI and periplasmic NapAB. Membrane-bound NR, with an active site in the cytoplasm, is coupled to the generation of transmembrane proton electrochemical gradient and is composed of three NarGHI subunits. The NarG subunit (104–150 kDa) contains the molybdopterin cofactor and is the site of nitrate reduction. The NarH subunit (43–63 kDa) is a globular protein containing iron-sulfur clusters. The last subunit, NarI (19–28 kDa) with two cytochrome b-type heme cofactors is a hydrophobic protein which constitutes the membrane domain of the enzyme (González PJ *et al.*, 2006a; Philippot & Højberg, 1999; Richardson *et al.*, 2001).

The respiratory NR found in the USDA 3045 strain was found to be predominantly membrane-associated, both nitrate- and chlorate-specific, and sensitive to micromolar concentrations of azide (Polcyn & Luciński, 2006). These features are hallmarks of NarGHI enzymes (Berks *et al.*, 1995; Bell *et al.*, 1990). However, in genomes of rhizobial strains sequenced so far, only information about the periplasmic type of dissimilatory NR has been found (Kaneko *et al.*, 2000; 2002; Galibert *et al.*, 2001; Delgado *et al.*, 2003; González *et al.*, 2006b). Therefore, unambiguous

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*Accession numbers: NarG protein: UniProt KB acc. no. P85097; NarH protein: UniProt KB acc. no. P85098.

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; MGD, molybdopterin guanine dinucleotide; NR, nitrate reductase; PIC, protease inhibitor cocktail, PMSF, phenylmethylsulfonylfluoride.

identification of the membrane-bound NR at the molecular level was needed. To achieve this, respiratory NR was purified from the USDA 3045 strain and its subunits were then identified by tandem mass spectrometry (MS/MS) amino acid sequencing. In addition, Western immunoblot screening with a polyclonal serum raised against membrane-bound NR, as well as a search for membrane-associated NR-active electrophoretic forms, were conducted to study the distribution of active NR of this type among other rhizobial species.

MATERIALS AND METHODS

Culture conditions and purification of NR. Eight-liter batches of USDA 3045 cultures, grown aerobically at 30°C in YM broth (Somasegaran & Hoben, 1994) to OD_{580} about 0.45, were spun and transferred to the fresh medium (4 l, OD_{580} about 0.85) supplemented with 4 mM nitrate. After 6 h of anaerobic incubation cells were harvested, washed and converted to spheroplasts according to Polcyn and Luciński (2006). Pelleted spheroplasts were suspended in 20 ml of 20% (v/v) glycerol in 50 mM Tris, pH 8, frozen in liquid nitrogen and stored at –20°C. Stored spheroplasts were supplemented with 3 mM EDTA and 10 μl/ml of PIC (protease inhibitor cocktail, Sigma-Aldrich P8849), thawed on ice and sonicated until the concentration of released proteins reached a plateau. After 60 min centrifugation at 240 000 × *g*, the membranes (about 10 mg of protein per ml^{-1}) were solubilized for 30 min, room temp., with 2% Triton X-100 in 0.1 M Tris, pH 8, containing 0.2 M NaCl, 11% (v/v) glycerol, 3 mM EDTA and 20 μl/ml of PIC.

For membrane-NR purification solubilized membrane proteins were dissolved in buffer A (1 mM PMSF, 5 mM benzamidine in 50 mM Tris, pH 8) to reduce the concentration of detergent to 0.4%, and purified by LC using a BioLogic LP chromatography system (Bio-Rad). A Macro-Prep High Q column (2.5×10 cm, Bio-Rad) equilibrated with buffer A containing 0.1% Triton X-100 and 0.15 M NaCl was developed with a two-step gradient (0.4 M and 1 M NaCl) of a flow rate of 8 ml/min. Fractions (60 ml total) at the beginning of the gradient were collected. Desalting and pH change was performed on a Sephadex G-25 column (130 ml, Sigma-Aldrich), with 0.1% Triton X-100 in 50 mM Tris, pH 7.5 (buffer B). Eluate was saturated with buffer B containing 0.8 M ammonium sulfate, applied on a HiTrap Phenyl FF (low sub) column (5 ml, Amersham Biosciences) equilibrated with the same buffer, and developed at a flow rate of 3 ml/min with ammonium sulfate in buffer B (0.8 M step and decreasing gradient from 0.6 M to 0.2 M). Fractions (14 ml total) within the conductance range from 91 to 80 mS/cm were collected, concentrated on a Centriplus YM-100 (Millipore) membrane to 1 ml, and applied to gel filtration column (HiPrep Sephacryl S-300 HR, Amersham Biosciences).

Mass spectrometry. Purified NR subunits were analyzed by nanoLC-electrospray-LTQ-FTICR mass spectrometry (Hybrid-2D-Linear Quadrupole Ion Trap–Fourier Transform Ion Cyclotron Resonance Mass Spectrometer, Thermo Electron Corp., San Jose, USA) in the Mass Spectrometry Laboratory, Institute of Biochemistry and Biophysics PAS, Warszawa, Poland. After 10% SDS/PAGE the gel bands containing purified NR subunits were excised, reduced with 100 mM DTT (for 30 min at 56 °C), alkylated with iodoacetamide (45 min in dark room, room temp.) and digested overnight with trypsin (Sequencing Grade Modified Trypsin; Promega V5111) according to Shevchenko *et al.* (1996). The resulting peptides were eluted from the gel with 0.1% trifluoroacetic acid and 2% acetonitrile. Peptide mixtures from each band were applied separately to an RP-18 precolumn (LC Packings) using water containing 0.1% trifluoroacetic acid as the mobile phase and then transferred to a nano-HPLC RP-18 column (nanoACQUITY UPLC BEH C18; Waters 186003545) using 0–60% acetonitrile gradient (30 min) in the presence of 0.05% formic acid at a flow rate of 0.15 μl/min. The column was coupled on-line to the electrospray ion source of the mass spectrometer working in the regime of data-dependent MS to MS/MS switch. A blank run ensuring lack of cross contamination from previous samples preceded each analysis.

Interpretation of the MS/MS-data. The output list of parent and daughter ions was used to search the database using the Mascot program (Matrix Science Ltd., London, UK). Acquired raw data were processed by Mascot Distiller followed by Mascot Search. The Mascot program was set to look in the NCBI non-redundant database for all the amino- acid sequences that show arginine or lysine at the C-terminus ("semiTrypsin" parameter) and match the measured parent ion masses of the tryptic peptides. Theoretical MS/MS mass spectra generated from such a selected database sequences were matched to empirical fragmentation spectra in the acquired data. The Mascot search parameters were set to: MS accuracy 40 ppm, MS/MS accuracy 0.8 Da, one missed cleavage site allowed, fixed carbamidomethyl modification of cysteine, and variable modification of oxidized methionine and carbamidomethylated lysine. The Mascot program reports an individual ion score for each assignment of a MS/MS spectrum to a database sequence and groups correctly identified peptides into sets according to their corresponding database-deposited proteins. The ion score is –10*Log(P), where P is the probability

($P<0.05$) that the observed match is a random event (Perkins *et al*., 1999). A threshold Mascot-score value, indicating identity or extensive homology, was calculated: at >55, for spectra matching NarG, and at >60 for NarH derivatives. The MS/MS fragmentation spectra due to double- and triple-charge states or covalent modifications of the same precursor ion mass provide enriched and complementary sequencing information. The sum of individual ion scores for peptide sets matching a particular database protein contributed to the identification of the MS/MS-analyzed NR subunits.

Assembling of partial protein maps of NarG and NarH. ClustalW 1.83 software (Lopez *et al*., 2003) and Jalview 2.3 editor (Clamp *et al*., 2004) were used to align the MS/MS-deciphered rhizobial amino-acid sequences with database NarG and NarH homologs of top Mascot-scoring values. Before assembling the partial protein maps of NarG and NarH additional validation criteria were applied to eliminate sequence uncertainty (Jensen *et al*., 1998; Perkins *et al*., 1999; Nesvizhskii & Aebersold, 2004; and references therein). (i) The sequences with Mascot scores <55 were rejected. (ii) Peptides must be fully tryptic, i.e. show arginine or lysine at both termini. (iii) More than 80% of the ions in the MS/MS spectra must be assigned to predicted amino acids. (iv) The spectrum must contain an uninterrupted series of b- or y-ions equivalent to at least five residues. (v) The average RMS error, a measure of the errors between values of empirical *m*/*z* of fragment ion spectra and theoretical ones, must be below 500 ppm. In several cases two or more matching sequences covered the same region of the alignment but differed in individual amino acid. The choice of which candidate homologous sequences to accept was based on: (i) quality of MS/MS spectra as defined above, (ii) the value of individual ion Mascot score, (iii) confirmation by additional fragmentation spectra, MS/MS-generated from the same precursor ion mass due to multiple charge state or covalent modifications.

Western immunoblotting and nondenaturing PAGE. Anaerobic induction of NR with nitrate and isolation of membrane proteins were done following the purification procedure described above. Triton X-100-solubilized membrane proteins (50 μg per lane) were separated by 4–15% gradient nondenaturing PAGE according to Polcyn and Luciński (2006). An optimized procedure ensuring efficient transfer and immunodetection of rhizobial membrane-associated proteins of molecular mass >120 kDa was developed. Sonicated cell extracts (20 μg of proteins per lane) were resolved on 7% polyacrylamide gels (Laemmli, 1970) and transferred onto Immobilon-P membrane (0.45 μm, Millipore) by wet transfer (500 mA, 3 h, with stirring and ice cooling) using 25 mM Tris, 192 mM glycine buffer with 0.01% SDS but without methanol. After transfer, blots were immersed in methanol, air-dried completely and treated according to the rapid immunodetection protocol from Millipore (2000). The IgG fraction of anti-NR_{II} serum (Polcyn & Luciński, 2006) was isolated by chromatography on Protein A–Sepharose CL-4B (Sigma-Aldrich) and used at 0.75 μg/ml concentration.

RESULTS AND DISCUSSION

Purification of rhizobial NR

Anaerobic rhizobial culture was grown with 4 mM nitrate for 6 h to reach a high induction level of predominating membrane-bound NR of 230 kDa. This enzyme was not inducible in aerobic culture with nitrate (Fig. 1). NR was purified from Triton X-100-solubilized membrane proteins by liquid chromatography on Macro-Prep High Q, HiTrap Phenyl FF (low sub) and HiPrep Sephacryl S-300 HR columns (see Materials and Methods). The 76-fold purified enzyme exhibited a specific activity of 35 μmol nitrite × (mg protein)$^{-1}$ × min^{-1}. Additional passage through a Resource Q column did not increase this value, therefore it seems to be the maximum specific activity of the rhizobial membrane-bound NR.

Such a purifed enzyme resolved by SDS/PAGE showed a homogenous α-subunit of 137-kDa molecular mass well correlating with the structure of bacterial membrane-bound NRs. Nevertheless, the β-subunit of the enzyme seemed to be degraded to some extent which resulted in the appearance of two bands of 65 and 59 kDa (Fig. 2).

The molecular mass of the purified native enzyme was determined by gel filtration (Fig. 3). Activity of the enzyme was found at Ve=62±1.7 ml,

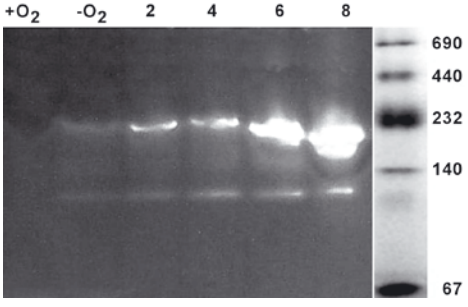

Figure 1. Induction of the membrane-bound NR-active 230-kDa complex from *Bradyrhizobium* sp. USDA 3045. Cells were anaerobically grown on 4 mM nitrate. Samples of Triton X-100-solubilized membrane proteins (50 μg) were taken at indicated intervals (hours), resolved on 4–15% gradient gel containing 0.2% Triton X-100 and in-gel stained for NR activity. $+O_2$: aerobic culture grown for 6 h with 4 mM nitrate, $-O_2$: anaerobic culture grown for 6 h without nitrate. Native HMW Calibration Kit (Amersham Biosciences) was used as protein standard.

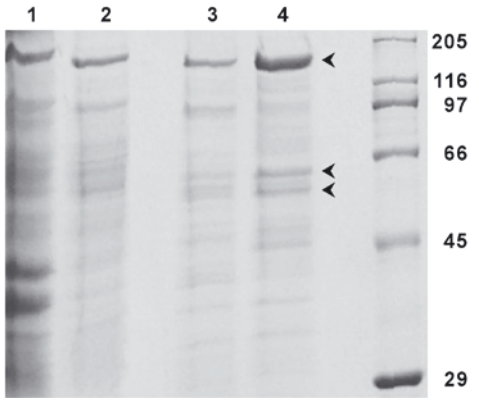

Figure 2. SDS/PAGE from various stages of purification of NR.
Lane 1, High Q, 30 μg; lane 2, Phenyl FF, 20 μg; lane 3, Sephacryl S-300 HR, 5 μg; lane 4, Resource Q, 10 μg. Stained with Coomassie brilliant blue R-250. The 137-kDa band (α-subunit) and two bands of 65-kDa and 59-kDa from lane 4 (arrows) were excised and subjected to MS/MS identification. High Molecular Weight Standard Mixture (Sigma-Aldrich) was used as protein standard.

corresponding to 180–220 kDa. This molecular mass was consistent with α1β1 subunit composition (202 kDa) and matched the average molecular mass of membrane-bound-type bacterial respiratory NRs (Berks *et al.*, 1995; Philippot & Højberg, 1999; Richardson *et al.*, 2001).

The electrophoretic mobility of the native enzyme (Fig. 1) was different from that expected for the 190-kDa value of the main membrane-bound NR complex indicated previously in non-denaturing gels from

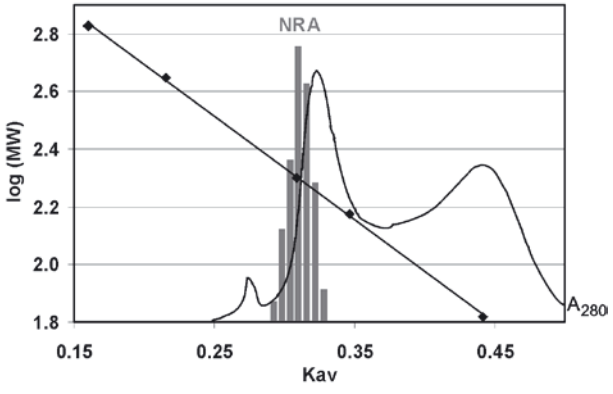

Figure 3. Estimation of molecular mass of *Bradyrhizobium* sp. NR by size-exclusion liquid chromatography.
The 0.5 ml sample of purified NR was loaded on HiPrep Sephacryl S-300 HR column. The eluted proteins were monitored at 280 nm. The calibration line was plotted against molecular masses of protein standards and $Kav=(V_e-V_0)/(V_t-V_0)$. Marker points indicate positions of standard proteins: thyroglobulin (669 kDa, V_e = 48.6 ml); apoferritin (443 kDa, V_e = 53.3 ml); β-amylase (200 kDa, V_e = 61.3 ml); alcohol dehydrogenase (150 kDa, V_e = 64.4 ml); BSA (66 kDa, V_e = 72.5 ml). Bars indicate fractions of the eluate containing nitrate reductase activity (NRA).

nitrate-respiring USDA 3045 strain (Polcyn & Lucinski, 2006). This can be explained by improvement in protection against proteolysis during cell disruption and enzyme solubilization, which resulted in a slower migration in the gel (see Materials and Methods). This can be seen especially for the higher 137-kDa molecular mass of the α-subunit, comparing to the 126-kDa value obtained previously. Such a proteolysis-related difference between separate isolations of respiratory NR from the same bacterial species has been reported already, for example, 112-kDa *versus* 130-kDa α NR subunit from *Pseudomonas stutzeri* (Blümle & Zumft, 1991; Hettmann *et al.*, 2003). Nevertheless, it should be noted that multiple NR-active electrophoretic forms of respiratory NR could occur in *Bradyrhizobium* sp. (*Lupinus*) cells, which might be immature complexes or derivatives of the enzyme protein turnover related to the level of nitrate available (Polcyn, 2008).

MS/MS identification of the 137-kDa NR α-subunit

The purified and SDS/PAGE-resolved 137-kDa α-subunit was excised and digested in-gel with trypsin. After digestion, the resulting peptide mixture was analyzed by nano-HPLC coupled to an LTQ FT ICR mass spectrometer. During this procedure, tryptic peptides converted by the electrospray ionization (ESI) source to multiply charged precursor ions were separated on the basis of the mass-to-charge ratio (*m*/*z*) and then subjected to low-energy collisional fragmentation. Since fragment ions are formed by cleavage of peptide bonds, their *m*/*z* values differ by corresponding amino-acid residue mass. This enables assignment of amino-acid sequences to the tryptic peptides (Perkins *et al.*, 1999; Ishihama, 2005; Medzihradszky, 2005).

The MS/MS ion spectra were evaluated by using the Mascot search engine against the bacterial non-redundant NCBI subdatabase (Feb. 07, 2006, 1 493 378 entries), according to Perkins *et al.* (1999). Electrospray ionization of tryptic peptides gave 299 ESI/MS precursor ions of monoisotopic mass values correctly matching *in-silico*-digested peptides from the NCBInr database. The observed fragment ion spectra of 277 Mascot queries matched the theoretical MS/MS spectra of database-deposited sequences of NarG, i.e. the α-subunit of bacterial respiratory NRs. These spectra represented 149 unique tryptic peptides, among them 93 had Mascot ion scores >55, indicating extensive homology (Perkins *et al.*, 1999) to database NarG proteins. Twelve top-scoring protein hits are shown in Table 1. These peptide sets had an overall sum of Mascot scores ranging from 4240 to 947, with a maximum protein sequence coverage of 47% and an RMS error below ±10 ppm. Note that within the 137-kDa electrophoretic band only a few other protein hits were detected, but with much lower Mascot

Table 1. Summary of MS/MS identification of the 137-kDa NR α-subunit

Accession number	Top significant protein hits	Mascot score[a]	Protein coverage (% aa)	No. of peptides matched[b]
gi|62180328	nitrate reductase 1, α-subunit (*Salmonella enterica* ssp. *enterica* sv. Choleraesuis str. SC-B67)	4240	47	64
gi|49329080	respiratory nitrate reductase, α-subunit (*Bacillus thuringiensis* sv. konkukian str. 97–27)	3266	45	54
gi|65319431	nitrate reductase α subunit (*Bacillus anthracis* str. A2012)	3092	44	60
gi|47566894	nitrate reductase, α subunit (*Bacillus cereus* G9241)	3083	45	59
gi|75242029	nitrate reductase α subunit (*Escherichia coli* F11)	3036	34	50
gi|29895803	respiratory nitrate reductase α chain (*Bacillus cereus* ATCC 14579)	2843	40	47
gi|56127733	respiratory nitrate reductase 2 α chain (*Salmonella enterica* ssp. *enterica* sv. Paratyphi A str. ATCC 9150)	1896	25	32
gi|49611485	respiratory nitrate reductase 1 α chain (*Erwinia carotovora* ssp. *atroseptica* SCRI1043)	1625	24	32
gi|77979848	nitrate reductase α subunit (*Yersinia intermedia* ATCC 29909)	1569	19	28
gi|75195571	nitrate reductase α subunit (*Escherichia coli* HS)	1551	19	27
gi|29652572	putative dissimilatory membrane-bound nitrate reductase (uncultured bacterium, partial sequence)	1022	71	15
gi|1009366	respiratory nitrate reductase (*Bacillus subtilis*)	947	14	18

[a]Protein scores derived from individual ion scores of Mascot-identified tryptic peptides as a non-probabilistic basis for ranking protein hits (Perkins *et al.*, 1999). [b]Total number of tryptic peptides which the Mascot program assigned to a database protein. To compute this number, multiple matches to rhizobial peptides with the same primary sequence but representing different charge or modification states were counted as one.

total scores (from 49 to 66), indicating that the purified α-subunit had a very high homogeneity. These data allowed unambiguous identification of the purified 137-kDa subunit of NR from USDA 3045 strain as a homolog of NarG-type proteins.

Assembling a protein map of rhizobial NarG with Mascot-identified sequences

The high amount of starting protein material greatly contributed to the number of MS/MS-sequenced tryptic fragments. This fact, as well as the high quality of empirical fragment ion spectra, allowed identification of the partial amino-acid sequence of *Bradyrhizobium* sp. NarG protein. Applying additional accuracy restrictions (see Materials and Methods), sequences of 64 individual peptides were selected with boosted confidence and were used to assemble a protein map of the subunit, basing on the alignment to two top-Mascot-scoring NarG homologs (Fig. S1 in the supplemental material: www.actabp.pl). The assigned 902 aa spanned 73% of rhizobial NarG protein, considering that the average length of 11 best-matched NarG database proteins is 1239 aa residues. This partial sequence was submitted to UniProtKB (acc. no. P85097).

MS/MS identification of the 65-kDa and 59-kDa NR subunits

Apart from the 137-kDa subunit, identified as a NarG-type protein, two additional SDS/PAGE bands of 65 kDa and 59 kDa were obtained as a result of the purification scheme used in this study. Both of them were subjected to MS/MS analysis and identified as derivatives of NarH, i.e. as the β-subunit of respiratory membrane-bound NRs (Table 2). The presence in purified NR preparation of two NarH-derived bands may reflect a higher susceptibility to proteolytic degradation of this subunit than it was in the case of NarG.

Tryptic peptides taken from both protein bands gave 42 MS/MS spectra matching the NarH database sequences. Eight protein hits, shown in Table 2, had an overall sum of Mascot scores ranging from 304 to 108, with maximum protein sequence coverage of 29% and an RMS error below ±8 ppm. The identified spectra represented 25 unique tryptic peptides. From them, 22 sequences were selected to establish a NarH partial protein map (Fig. S2 in supplemental material: www.actabp.pl). The 266 aa which were allocated (UniProtKB acc. no. P85098) constituted about 52% of rhizobial NarH, assuming that the average length of bacterial NarH database proteins is 511 aa.

Analysis of rhizobial NarG and NarH protein sequences

When the deciphered amino-acid sequences of rhizobial NarG and NarH proteins were aligned with bacterial homologs of the highest total Mascot scores, extended regions of identity were identified (Figs. S1 and S2 in supplemental material:

Table 2. Summary of MS/MS identification of 65-kDa and 59-kDa NR subunits

Accession number	Top significant protein hits	Mascot score[a]	Protein coverage (% aa)	No. of peptides matched[a]
	65-kDa band:			
gi|16129188	nitrate reductase 1, β-(Fe-S) subunit (*Escherichia coli* K12)	304	29	12
gi|16760301	respiratory nitrate reductase 2 β chain (*Salmonella enterica* ssp. *enterica* sv. Typhi str. CT18)	285	29	13
gi|15801671	cryptic nitrate reductase 2, β subunit (*Escherichia coli* O157:H7 EDL933)	228	26	12
	59-kDa band:			
gi|16129188	nitrate reductase 1, β (Fe-S) subunit (*Escherichia coli* K12))	283	23	10
gi|16760301	respiratory nitrate reductase 2 β chain (*Salmonella enterica* ssp. *enterica* sv. Typhi str. CT18)	265	24	11
gi|16765104	nitrate reductase 1 β subunit (*Salmonella typhimurium* LT2)	252	28	12
gi|91769211	nitrate reductase, β subunit (*Psychrobacter* sp. PRwf-1))	164	19	8
gi|7321241	nitrate reductase β-subunit (*Pseudomonas* sp. Ki-1z)	108	8	2

[a]For details, see Table 1

www.actabp.pl). As expected from its phylogenetic positions, *Bradyrhizobium* sp. NarG showed a higher sequence identity (WU-BLAST2, Lopez *et al.*, 2003; parameters: blastp, database: UniProtKB, matrix: blosum62) to *Escherichia coli* and *Salmonella enterica* (79%) than to the taxonomically more distant *Bacillus thuringiensis* (53%). In the case of rhizobial NarH these values were respectively: 71% and 58%.

Prokaryotic NarG is a member of a superfamily of oxidoreductase subunits containing a molybdo-bis(molybdopterin guanine dinucleotide) (Mo-bisMGD) cofactor (Berks *et al.*, 1995; Bertero *et al.*, 2003; Jormakka *et al.*, 2004). The assembled partial sequence of the rhizobial NarG exhibited most of the conserved segments which had been implicated in MGD binding and in the electron transfer for nitrate reduction and constituting individual domains of Mo-bisMGD subunits (Fig. S1 in supplemental material: www.actabp.pl) (Blasco *et al.*, 2001; Jormakka *et al.*, 2004). An exception was the missing fragment of domain III, which provides a molybdenum-coordinating Asp residue (Asp222 in *E. coli*) highly conserved in NarG and other subunits of type II group of Mo-bisMGD enzymes (Bertero *et al.*, 2003; Jormakka *et al.*, 2004). Presumably, it was not detected by MS/MS in the rhizobial NarG due to a lack of trypsin-specific sites in the surrounding sequence region. Nevertheless, an almost complete His/Cys cluster (His49, Cys53, Cys57, Cys92 in *E. coli*) coordinating a [4Fe-4S] group and highly conserved in NarG subunits, as well as all conserved groups of cysteine residues (except Cys217 of *E. coli*), thought to bind one [3Fe-4S] and three [4Fe-4S] clusters in NarH subunits (Blasco *et al.*, 2001; Jormakka *et al.*, 2004), were found in the aligned rhizobial sequences (Figs. S1 and S2 in the supplemental material: www.actabp.pl).

A similarity search was also conducted among the deduced protein sequences from the RhizoBase — a database compiled from several complete rhizobial genomes (Nov. 8, 2007, http://bacteria.kazusa.or.jp/rhizobase). WU-BLAST2 search showed 61% aa identity of NarG from USDA 3045 to the hypothetical NarG-type NR α-subunit from *Azorhizobium caulinodans* ORS 571 (acc. no. YP_001524341). Slightly higher level of similarity (64–69%) was found for NarG-type proteins from several species belonging to the genera *Burkholderia* and *Ralstonia*. Recently, some nodule bacteria were classified into this genera (Willems, 2006). The amino-acid sequence identity of NarH from *Bradyrhizobium* sp. USDA 3045 to β NR subunits from the above-mentioned species was 63–65%.

Distribution among other rhizobial species of active membrane-bound NR forms immunologically related to the α NR subunit from *Bradyrhizobium* sp. USDA 3045

In order to study whether other rhizobia could express membrane-bound NR, nine additional strains, representing six species and three rhizobial genera (listed in Table 3), were anaerobically induced with 4 mM nitrate and screened by Western blotting with anti-NR_{II} polyclonal serum. In parallel, searches for membrane-associated NR-active electrophoretic forms were conducted, resolving Triton X-100-solubilized membrane proteins from these strains by nondenaturing-PAGE and detecting the NR activity directly in-gel.

The anti-NR_{II} antibodies were raised previously against the slower-migrating native electrophoretic form of purified membrane-bound NR from the USDA 3045 strain (Polcyn & Luciński, 2006). Presently, the immunospecificity of this serum was confirmed conclusively by binding of anti-

Table 3. Bacterial strains used in this study

Acronym	Strain	Host plant	Origin	Source
Blu	*Bradyrhizobium* sp. USDA 3045	*Lupinus luteus*	USA	USDA
Bl3	*Bradyrhizobium* sp. JZ 3.1.3	*Lupinus luteus*	Poland	ZBB AU
Bl4	*Bradyrhizobium* sp. JZ 4.2.1	*Lupinus luteus*	Poland	ZBB AU
Bl5	*Bradyrhizobium* sp. JZ 5.2.1	*Lupinus luteus*	Poland	ZBB AU
Mam	*Mesorhizobium amorphae* ICMP 15022	*Amorpha fruticosa*	China	ICMP
Mlo	*Mesorhizobium loti* USDA 1129	*Lotus corniculatus*	USA	USDA
Ret	*Rhizobium etli* USDA 9032	*Phaseolus vulgaris*	USA	USDA
Rt24	*Rhizobium leguminosarum* bv. *trifolii*: 24	*Trifolium* sp.	Poland	ZMO UMCS
RvL	*Rhizobium leguminosarum* bv. *viciae*: L	*Lathyrus sativus*	Poland	ZMR IUNG
RvS	*Rhizobium leguminosarum* bv. *viciae*: ST	*Lens culinaris*	Poland	ZMR IUNG
Pst	*Pseudomonas stutzeri* DSM 5190	--	Germany	DSM

DSM: German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; **ICMP**: International Collection of Microorganisms from Plants, Landcare Research, Auckland, New Zealand; **USDA**: United States Department of Agriculture, Beltsville; **ZBB AU**: Department of Biochemistry and Biotechnology, August Cieszkowski Agricultural University, Poznań, Poland; **ZMO UMCS**: Department of General Microbiology, Marie Curie-Skłodowska University, Lublin, Poland; **ZMR IUNG**: Department of Agricultural Microbiology, Institute of Soil Science and Plant Cultivation, Puławy, Poland.

NR_{II} IgGs to MS/MS-identified α and β-subunits of purified NR (Fig. 4a, Blu). Furthermore, a competition-based adsorption assay was carried out by incubation of the serum with the purified NR before immunostaining. As a result, the immunoreactive bands were no longer detected by the pre-absorbed antiserum in crude extracts from all rhizobial strains tested (not shown). In addition, an immunoassay of total protein extract from nitrate-induced reference denitrifying species *Pseudomonas stutzeri* (contains NarGH enzyme, Blümle & Zumft, 1991; Hettmann *et al*., 2003) showed a very strong signal with anti-NR_{II} antibodies at 65 kDa, corresponding to the relative mobility of the β NR subunit from USDA 3045. Immunostaining with a pseudomonal α NR subunit was weaker but also visible (Fig. 4a, Pst).

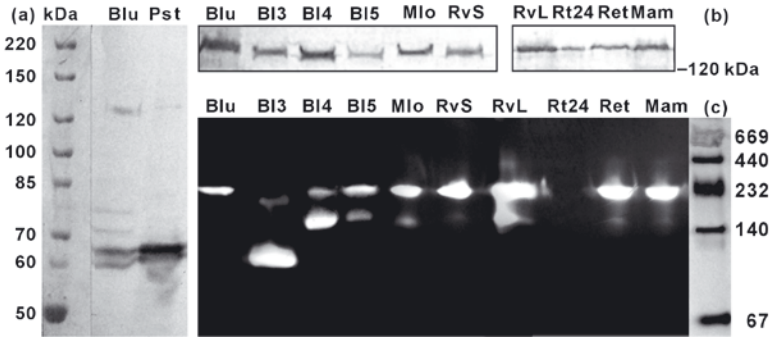

Figure 4. Detection of NR-active membrane-bound electrophoretic forms immunologically related to NarGH-type NR from USDA 3045.
(a) Immunospecificity of anti-NR_{II} IgGs against: (Blu) MS/MS identified alpha and beta subunits of NR purified from USDA 3045 and (Pst) total protein extract from *Pseudomonas stutzeri* DSM 5190. (b) Western immunoblot screening of various rhizobial strains with anti-NR_{II} IgGs. (c) Non-denaturing PAGE of membraneous proteins from rhizobial strains and in-gel NR activity staining. Lanes were marked with acronyms of the strains described in Table 3. All strains were anaerobically induced with 4 mM nitrate for 3 h (*P. stutzeri*) or 4–6 h (rhizobia) until nitrite production reached the maximum.

Figure 4b demonstrates the results of the Western immunoblot screening of ten rhizobial strains with anti-NR_{II} IgGs. Surprisingly, a NarG-like protein was recognized in all these bacteria and they exhibited very similar molecular masses. All of these strains expressed also a NarH-like protein, however, it could not be visualized on the same blots due to much faster migration during wet transfer optimized for proteins of molecular mass >120 kDa.

Nondenaturing PAGE of membraneous proteins and in-gel activity staining revealed NR-activity bands of similar electrophoretic mobility in nine of the tested strains (Fig. 4c). The 230-kDa molecular mass of these bands corresponded well to the electrophoretic mobility of the main NR form found in USDA 3045, although in some strains more rapidly migrating protein bands were also observed, possibly representing proteolytic degradation products. The investigated strains dissimilated up to 75% of the initially added 4 mM nitrate within 3–4 h of anaerobic culture (not shown). This rate was, however, two-fold slower than nitrate reduction in the reference denitrifying strain *P. stutzeri* DSM 5190. It should be noted that rhizobial cultures were nitrate-induced at the high cell density of OD_{580} about 0.85.

To my knowledge such a comparison of various rhizobial strains has not been performed before. Nitrate-respiring strains of other genera have been found to contain both periplasmic NapAB and membrane-bound NarGHI, and some others exhibited only one type of NR (Philippot & Højberg, 1999; Richardson *et al*., 2001; González *et al*., 2006a).

Considering this, to study the distribution of respiratory NR among rhizobial species, more conclusive investigations, on a wider representation of rhizobial strains and nodule isolates are needed with *NapA*- and *NarG*-specific DNA hybridization probes.

Acknowledgements

I am grateful to Prof. Wanda Małek, Prof. Cezary Mądrzak and Dr. Joanna Króliczak for providing the strains used in this study. I also thank Dr. Janusz Dębski for help in interpretation of the MS/MS data.

This work was supported by grant 2 P06R 077 27 from the State Committee for Scientific Research.

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