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Respiratory nitrate reductase from denitrifying *Pseudomonas stutzeri*, purification, properties and target of proteolysis

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Respiratory nitrate reductase (EC 1.7.99.4) from the denitrifying bacterium *Pseudomonas stutzeri*, strain ZoBell (ATCC 14405), was purified by a rapid three-step procedure of differential centrifugation, DEAE-Sepharose chromatography and gel filtration. The enzyme was released from the cytoplasmic membrane by heat. Purification was about 25-fold to an average specific activity of 27 μmol nitrate reduced per min per mg protein. Non-denaturing electrophoresis in the presence of 1% Triton X-100 resolved two catalytically active enzyme species with apparent M_r 140000 and 132000. The two forms had each a 112 kDa subunit and one or two types of small subunit (60 or 46 kDa). The membrane-bound enzyme was composed of two subunits of M_r 112000 and 60000 as shown by Western blot analysis. The subunit heterogeneity was caused by an endogenous proteinase which modified the small subunit. Nitrate reductase contained per M_r 172000 about 13 iron-sulfur groups and one gatom of molybdenum bound to a pterin cofactor. The electronic spectrum of the purified enzyme had a weak maximum at 410 nm; however, there was no spectral evidence for a cytochrome moiety. The K_m value for nitrate was 3.8 mM. Azide and cyanide were inhibitory for nitrate reductase with K_i values of 0.7 μM and 0.1 mM, respectively. Addition of 0.1 mM azide to the growth medium increased the cell-free enzyme activity 4-fold, due to a concomitant overproduction of nitrate reductase.

Introduction

Denitrification is an alternative way of energy conservation of facultative anaerobic bacteria. Nitrate reductase initiates a complete denitrification pathway which involves four individual reaction steps. There is a regulatory interaction among these steps, with nitrate reduction believed to play a central role. The respiratory enzymes, nitrite reductase (cytochrome cd_1, 1.9.3.2) [1], nitric oxide reductase (presently no valid EC entry) [2], and nitrous oxide reductase (EC 1.7.99.6) [3], but not yet nitrate reductase, have been purified from *Pseudomonas stutzeri* with the objective to characterize in its entirety the denitrification process of a single bacterial species.

Respiratory nitrate reductase (EC 1.7.99.4) is known best from *Escherichia coli*, a non-denitrifying bacterium. It consists of two subunits with molecular masses of 150 and 60 kDa [4–6]. The detergent-solubilized enzyme often has a third, heme-*b*-containing subunit of approx. 20 kDa [7–10]. This enzyme is coded for by the *narGHJI* operon [6]. Prosthetic groups of nitrate reductase are 3Fe and several 4Fe iron-sulfur clusters [11] and a pterin cofactor for molybdenum [12]. The enzyme is subject to limited proteolysis during heat solubilization [13]. Recent advances indicate that *E. coli* has a second membrane-bound nitrate reductase [14]. This enzyme has largely similar properties, but is encoded by the *narZ* gene, which is expressed aerobically and is not regulated by the FNR protein.

Respiratory nitrate reductases of denitrifying bacteria have been characterized in much less detail [15,16]. The genus *Pseudomonas*, in its ribosomal RNA homology group I, represents one of the largest assemblies of denitrifying bacteria within a single taxon. Here we describe the isolation of nitrate reductase from *P. stutzeri*, study its modification during purification, and report several basic properties.

Experimental procedures

Media and growth conditions. Pseudomonas stutzeri strain ZoBell (ATCC 14405) was grown anaerobically in

Abbreviations: SDS, sodium dodecyl sulfate; PAB, *p*-aminobenzamidine.

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a synthetic asparagine, citrate, and nitrate-containing medium (ACN) [17]. For growth in the presence of azide, 200 ml ACN medium was made 0.05, 0.1 or 0.2 mM in NaN_3 and was inoculated with 20 ml of an overnight culture. Growth flasks were incubated at 30°C on a rotary shaker (120 rev/min) to give oxygen-limited growth conditions. The cells were harvested by centrifugation at $1040 \times g$. The pellet was washed twice with 10 ml of 20 mM potassium phosphate buffer (pH 7.2), and suspended in 10 ml of this buffer. The *Pseudomonas* species to be tested for immunochemical cross-reactivity were grown anaerobically in Luria-Bertani broth (pH 7), containing 1 g $NaNO_3$ per liter. Cells were washed after harvest in 50 mM phosphate buffer (pH 7). Bacterial strains were obtained, as indicated, from the DSM (Braunschweig, F.R.G.), the ATCC (Rockville, MD, U.S.A.), or the ICPB (Davis, CA, U.S.A.) culture collections.

Neurospora crassa mutant strain *nit-1* (a gift from O. Meyer, Bayreuth, F.R.G.) was grown in liquid culture on Fries basal medium [18].

Preparation of cell extracts and membranes. The membrane fraction was isolated from *P. stutzeri* as previously described for the isolation of nitric oxide reductase [2]. Cell extracts for individual assays were prepared by sonicating cell suspensions for 20 min with a Branson B15 instrument, operating at an output level 4 and a pulse rate of 20%. Unbroken cells were removed by centrifugation. Induction of assimilatory nitrate reductase of *Neurospora crassa nit-1* and preparation of extracts for the molybdenum cofactor assay were as described in Ref. 12.

Purification of nitrate reductase. The membrane suspension was adjusted to a protein concentration of about 10 mg/ml with 20 mM potassium phosphate buffer (pH 7.2) (buffer A). It was heated in a water bath for 1 h at 60°C under continuous stirring and then rapidly cooled in ice/water. Membranes were collected by centrifugation at $186\,400 \times g$ for 90 min and 4°C. The supernatant contained the solubilized nitrate reductase. The following chromatographic steps were carried out in the cold.

The enzyme was applied to a column (11×2.5 cm) of DEAE-Sepharose CL-6B (Pharmacia-LKB, Freiburg, F.R.G.), equilibrated with 20 mM buffer A. The column was developed with 400 ml of a linear, phosphate-buffered gradient of zero to 0.5 M NaCl. Fractions that contained nitrate reductase activity were pooled and concentrated to about 2 ml by ultrafiltration (XM-50 membrane; Amicon, Witten, F.R.G.). The concentrated pool was further purified on a Sephadex G-150 column (1.7×90 cm); elution was with 20 mM buffer A. Nitrate reductase-containing fractions were concentrated as above and stored at −24°C.

The two proteolytically modified forms of nitrate reductase were separated by electrophoresis as follows. Samples of 4 mg of the enzyme from Sephadex G-150 chromatography were made 1% in Triton X-100 and incubated for 1 h on ice. The samples were applied to a 1 mm horizontal slab gel of a linear polyacrylamide gradient of 3 to 13% containing also 1% Triton X-100. The electrophoresis was initially run at 250 V and 4°C; after 15 min the voltage was set to 500 V for the remaining 3–4 h. The electrophoresis buffer was 14 mM Tris and 192 mM glycine (pH 8.3) [19].

The two brownish bands were identified after electrophoresis by an enzyme stain (cf. assays) as nitrate reductases. Each band was cut from the gel, homogenized and eluted at 200 V in a Biotrap (Schleicher & Schuell, Dassel, F.R.G.) according to the instructions of the manufacturer. Elution buffer was the Tris-glycine electrophoresis buffer [19]. The enzyme was concentrated by ultrafiltration (Centricon microconcentrator; Amicon, Witten, F.R.G.) to about 200 µl, frozen in liquid nitrogen, and stored at −24°C.

Trypsin treatment. Nitrate reductase (2.5 mg/ml) from Sephadex G-150 filtration or from non-denaturing electrophoresis was mixed with an equal volume of trypsin (5 mg/ml 1 mM HCl) from bovine pancreas (Boehringer Mannheim, F.R.G.) and incubated at 30°C for 30 min [20]. Samples were treated either with 1% SDS and 0.01% dithioerythritol at 80°C and subjected immediately to SDS-polyacrylamide gel electrophoresis, or were made 1% in Triton X-100 for non-denaturing separations and subjected to electrophoresis after 1 h of incubation on ice.

Extraction of the molybdenum cofactor and complementation assay. Nitrate reductase (3.4 mg/ml) in 100 mM phosphate buffer (pH 7.3), containing also 20 mM Na_2MoO_4, was denatured at 100°C for 30 s. The mixture was cooled on ice and centrifuged at $10\,000 \times g$ for 10 min. The supernatant was used for the complementation assay [12]. This assay contained in 300 µl of total volume, 50 µl of cofactor solution, 50 mM potassium phosphate buffer (pH 7.3), 1 mM EDTA, 20 mM $NaMoO_4$ and 100 µl extract of strain *nit-1*. The mixture was incubated at 30°C for 20 min. Samples of 50 µl were assayed for NADPH nitrate reductase activity.

Enzyme assays. Nitrate reductase activity was measured at 30°C by a modified method of Lowe and Evans [21]. The complete reaction mixture contained in a total volume of 1 ml, 40 µmol of buffer A, 20 µmol KNO_3, 0.4 mg methyl viologen, and an enzyme sample. The reaction was started by the addition of 100 µl of a freshly made solution of 50 mM dithionite in 0.1 M $NaHCO_3$. The reaction was stopped by rapid air oxidation of the viologen. Samples of 0.1 ml were assayed for nitrite [22]. NADPH nitrate reductase of *Neurospora* extracts was measured as described in Ref. 23. Pyridine nucleotides were precipitated by barium acetate/ethanol before assaying for nitrite [18]. One unit (U) of methyl viologen or NADPH-dependent nitrate reductase activ-

ity is the amount of enzyme that catalyzes the reduction of 1 μmol substrate per min. Molybdenum cofactor activity was measured as reconstituted nitrate reductase activity in the complementation assay and is expressed as nmol nitrite produced per min [24]. Nitrate reductase activity in polyacrylamide gels was detected by staining gels for 10 min with 10 mM methyl viologen in 50 mM buffer A containing 2 mg/ml dithionite [4]. Immersion of a stained gel in 200 mM nitrate solution caused an immediate decolorization at the position of nitrate reductase. Agarose gels were washed first in 0.9% NaCl, rinsed in H_2O, and then treated as polyacrylamide gels.

Immunochemical methods. Antibodies against nitrate reductase were raised in New Zealand white rabbits. Electrophoretically homogeneous enzyme (0.5 mg) was emulsified with Freund's complete adjuvant and injected subcutaneously. Boosting injections of 0.25 mg protein in Freund's incomplete adjuvant were made after 4, 6 and 8 weeks. Blood samples were taken after 4, 7 and 9 weeks. The antibody titer was followed by Ouchterlony double immunodiffusion [25]. Pre-immune sera showed no cross-reactivity with nitrate reductase. The antiserum was tested for monospecificity by crossed immunoelectrophoresis [26].

Quantitation of nitrate reductase in crude extracts and membrane fractions was done by rocket immunoelectrophoresis [26] in 1 mm gels of Agarose NA (Pharmacia-LKB, Freiburg, F.R.G.) containing 1% antiserum and 1% Triton X-100. For Western-blot analysis, proteins were transferred electrophoretically from polyacrylamide gels onto nitrocellulose [26]. Nitrate reductase was detected by immunoblotting and peroxidase-staining with 4-chloro-1-naphthol as chromogen [28].

Analytical methods. Iron was determined by atomic absorption spectroscopy at 248.3 nm. Molybdenum was assayed with the dithiol reagent [29], and acid-labile sulfur by the method of Beinert [30]. Protein was estimated by the Lowry method. Optical absorption spectra were recorded with a Shimadzu UV-260 spectrophotometer. For chemical reduction a few crystals of sodium dithionite were added. SDS-polyacrylamide gel electrophoresis was run in a linear gradient of 9–22% acrylamide [19]. Protein samples containing 0.01% dithioerythritol and 1% SDS were heated for 5 min at 80 °C. Gels were stained with Coomassie brillant blue R-250. The isoelectric point was determined with a surface electrode after focusing the protein in a Servalyte gradient, pH 3–9 [19], and detecting the enzyme by the activity stain. Molecular mass protein standards were purchased from Pharmacia-LKB, Freiburg, F.R.G.

Results and Discussion

Purification and heat-induced release of nitrate reductase

Nitrate reductase was solublized from the cytoplasmic membrane by heat under conditions similar to that developed for *P. aeruginosa* [31]. Approx. 50% of the enzymatic activity, but only 20% of membrane proteins was solubilized in medium scale preparations with a membrane protein concentration of about 10 mg/ml. The enzyme was purified by a rapid three-step procedure consisting of differential centrifugation, DEAE-Sepharose CL-6B chromatography, and gel filtration on Sephadex G-150. Ion-exchange chromatography was the most effective step of purification. The average specific activity of the enzyme after gel filtration was 27 U (maximum 31 U) per mg protein, representing a 25-fold purification over the intact membrane and a yield of about 20%. Table I shows the results of a representative purification protocol. In comparison to the procedure for *P. aeruginosa* nitrate reductase [31], the method described here for *P. stutzeri* is much simpler, and yields a specific activity of the enzyme about 10-fold higher and significantly more nitrate reductase protein per batch.

Whether proteolysis was required for heat-induced release of nitrate reductase from the membranes was probed with the serin proteinase inhibitor, PAB [9]. A washed membrane suspension was divided into 1-ml samples. PAB from a neutralized stock solution (50 mg/ml) was added to give the desired final concentrations. The samples were incubated and assayed as described in Table II. Involvement of a proteinase for solubilization was indicated by the decrease in the amount of nitrate reductase released in the presence of increasing amounts of PAB. However, the *E. coli* nitrate reductase can be dissociated from the membrane also without proteolysis [32]. Heat treatment increases the activity of certain respiratory nitrate reductases [9,31]. This was not the case with the *P. stutzeri* enzyme. Table II shows that heating in fact decreased the enzyme activity by about 20%.

Molecular mass and subunit composition

Non-denaturing polyacrylamide gel electrophoresis of nitrate reductase after gel filtration resolved two enzymatically active forms with apparent M_r of 140 000 (form I) and 132 000 (form II). Resolution of both forms required the presence of 1% Triton X-100 during electrophoresis (Fig. 1). Without Triton X-100 no discrete migration of protein bands was achieved. The

TABLE I

Purification of respiratory nitrate reductase from P. stutzeri

Fraction	Vol. (ml)	Protein (mg)	Activity (U)	Sp. act. (U/mg)	Yield (%)
Membranes	420	7140	8144	1.14	100
Supernatant 186 400 × *g*	300	1350	3862	2.86	47
DEAE-Sepharose CL-6B	40	131	2792	21.31	34
Sephadex G-150	42	65	1753	26.97	22

TABLE II

Effect of p-aminobenzamidine on heat solubilization of P. stutzeri respiratory nitrate reductase

Samples of a washed, diluted membrane suspension (about 1 mg protein/ml) in 20 mM phosphate buffer (pH 8) from anaerobically nitrate-grown cells were incubated for 90 min at room temperature with increasing amounts of PAB. The membrane suspensions were then subjected to heat treatment (1 h, 60°C) and centrifuged at 111000 × *g*. The percentage of enzyme released was related to the activity of the original membrane suspension.

PAB (mM)	Nitrate reductase activity (μmol NO_2^- formed per min)			
	membrane suspension	heated membrane suspension	supernatant of heated suspension	enzyme released (%)
–	1.53	1.19	1.14	74
1	1.53	1.19	1.11	72
10	1.61	1.21	0.88	56
40	1.48	1.08	0.69	46

relative amount of form II ranged in several independent preparations from equimolarity with form I, to an about 2.5-fold excess. Trypsin treatment converted the two forms to a single, electrophoretically homogeneous

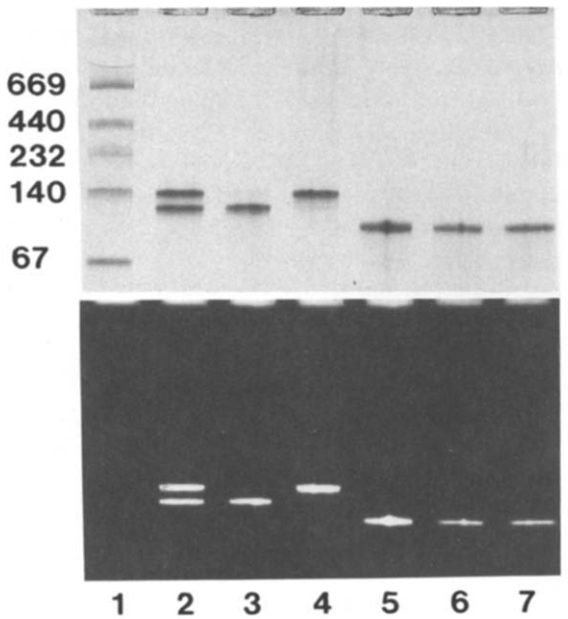

Fig. 1. Resolution of nitrate reductase into two enzymatically active forms, and conversion into a 95 kDa species by trypsin. Enzyme samples were analyzed by non-denaturing polyacrylamide gel electrophoresis in the presence of 1% Triton X-100. Upper panel: lane 1, standard proteins thyroglobulin, ferritin, catalase, lactate dehydrogenase, and albumin; molecular masses in kDa; lane 2, nitrate reductase preparation (11 μg protein) after Sephadex G-150 filtration showing the two enyzme species; lanes 3 and 4, enzymes form II (7 μg protein) and form I (6 μg protein), respectively, after preparative electrophoretic separation; lanes 5, 6 and 7, enzyme preparations as in lanes 2 through 4 after trypsin treatment. Staining by Coomassie blue. Lower panel same as upper panel, but activity stain for nitrate reductase.

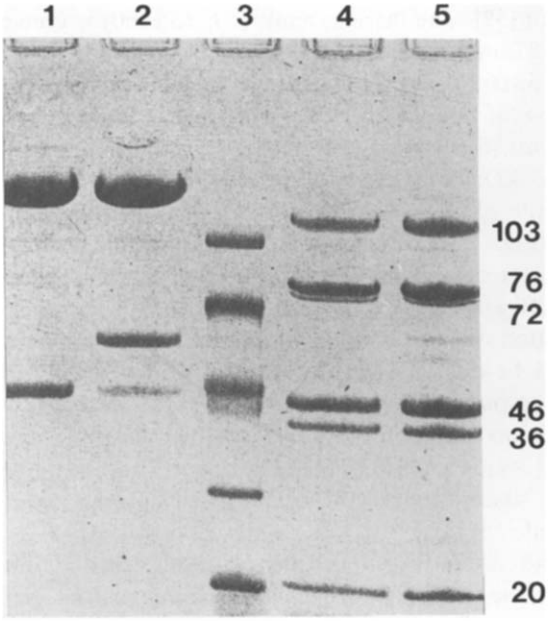

Fig. 2. Subunit composition of the two forms of *P. stutzeri* nitrate reductase as isolated, and after treatment with trypsin. Samples were analyzed by SDS electrophoresis and stained with Coomassie blue. Lane 1, enzyme form II (undissociated apparent mass 132 kDa); lane 2, enzyme form I (undissociated apparent mass 140 kDa); lanes 4 and 5, forms II and I, respectively, after trypsin treatment (cf. Experimental procedures). Bands due to trypsin ran below the 20 kDa band. Lane 3, protein standards as in Fig. 3. Numbers indicate in kDa the mass of nitrate reductase fragments.

species with lower molecular mass (approx. 95 kDa), which retained catalytic activity (Fig. 1A and B). Conversion of the two forms of nitrate reductase into a single species indicated that both might be of common origin. This notion was supported by the finding of an identical peptide pattern of the two forms after trypsin treatment.

Trypsin fragmented the enzyme into six major peptides ranging in apparent mass in SDS electrophoresis from approx. 20 to 103 kDa (Fig. 2, lanes 4 and 5). The fragments generated from the two subunits remained immunoreactive with a polyclonal nitrate reductase antiserum. Despite of this proteolysis, trypsin-treated nitrate reductase migrated in a non-denaturing, Triton-containing electrophoresis gel uniformly as a protein complex of 95 kDa (see Fig. 1A). As stated above, even the native enzyme did not migrate uniformly without detergent. We found that the nitrate reductase complex in the presence of Triton has in non-denaturing electrophoresis a much higher mobility (equivalent to M_r 140000) than expected from the sum of its subunits (equivalent to M_r 172000). Also, the 95 kDa complex observed in non-denaturing electrophoresis showed under denaturing conditions a fragment of 103 kDa (cf. Figs. 1 and 2).

Trypsin affects both subunits of the nitrate reductase of '*P. denitrificans*' in a similar way, though to a lesser

extent [33]. The large subunit (M_r 136000) is converted into 87 and 47-kDa peptides, whereas the small subunit (M_r 55000) is converted into a 47 kDa peptide. Modification of the *E. coli* enzyme involves only the small subunit, this being reduced in molecular mass from 59 to 43 kDa [32]. However, both subunits of the NarZ enzyme of *E. coli* are susceptible to trypsin [14]. The 140 kDa subunit of the cytoplasmic enzyme of the nitrate respirer *Staphylococcus aureus* is modified to a 112 kDa species by trypsin. Because this enzyme preparation was not homogeneous, no definitive conclusion could be drawn with respect to the small subunit [20]. The respiratory nitrate reductase from *Paracoccus denitrificans* apparently is not modified during purification [34]. It consists of 127, 61 and 21 kDa peptides; their susceptibility towards trypsin has not been reported.

The subunit composition of either form of nitrate reductase as isolated was analyzed by SDS electrophoresis (Fig. 2). Both forms had a 112 kDa polypeptide. Form I was a heterodimer with part of the 60 kDa subunit degraded to 46 kDa; in form II the small subunit was completely modified to the 46 kDa species. The cleaved fragment of about 14 kDa was detected in electrophoretic patterns of form I inconsistently, because it stained weakly and often had a low concentration.

The subunit composition of nitrate reductase in situ was established by immunoblotting (Fig. 3). The antiserum against the holoenzyme reacted also with the smaller peptides and was apt to identify proteolytic fragments. The electrophoretically resolved polypeptide pattern of isolated membranes was probed before and after heat treatment. Without heating, we found two nitrate reductase subunits of 112 and 60 kDa. The 46 kDa peptide appeared only after heat treatment (Fig. 3), proving that nitrate reductase was susceptible to heat-induced proteolytic modification involving the small subunit.

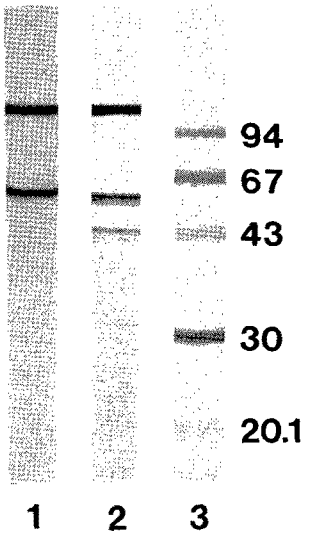

Fig. 3. Immunoblot analysis of the subunit composition of *P. stutzeri* nitrate reductase in situ and after heat solubilization. The membrane samples were subjected to SDS electrophoresis and analyzed by immunoblotting. Lane 1, membrane suspension as isolated; lane 2, membrane suspension subjected to heat treatment (60°C, 1 h); lane 3, Ponceau S-stained standard proteins, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor; molecular masses in kDa.

TABLE III

Chemical composition of respiratory nitrate reductase from P. stutzeri

Contents of nonheme iron, acid-labile sulfur, and molybdenum from independent preparations were calculated for M_r 172000. Iron was estimated by atomic absorption spectroscopy; molybdenum and acid-labile sulfur by chemical methods as specified under Experimental procedures.

Expt.	Fe	S^{2-}	Mo
1	12.5	12.1	0.85
2	12.7	8.7	0.49
3	12.8	10.2	0.49

Enzyme properties

Heat-solubilized nitrate reductase of *P. stutzeri* has a K_m value for nitrate of 3.8 mM with reduced methyl viologen as the electron donor. The enzyme was active also with benzyl viologen. Cyanide and azide were strong inhibitors and showed with nitrate competitive inhibition patterns. The K_i values were 0.7 μM for azide, and 0.1 mM for cyanide. The isoelectric point of the enzyme was pH 5.6.

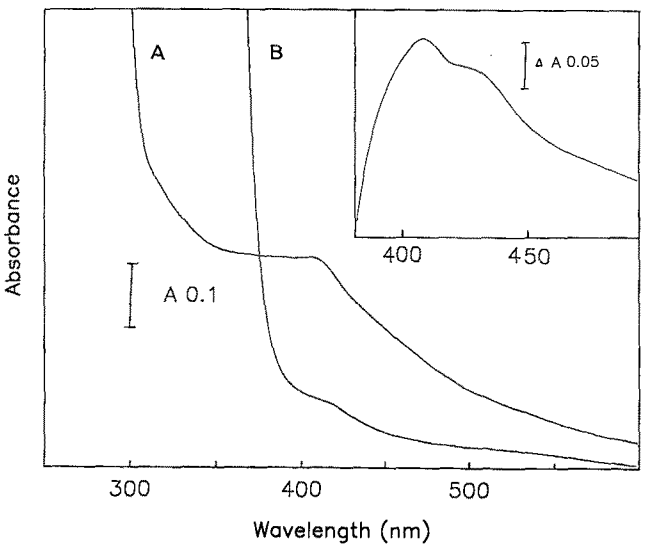

Fig. 4. Absorption spectra of purified nitrate reductase from *P. stutzeri*. (A) Enzyme as isolated (1 mg/ml in 20 mM phosphate buffer (pH 8); (B) the same sample reduced by dithionite. Inset, oxidized-minus-reduced difference spectrum. Light-path 1 cm.

Analysis by atomic absorption spectroscopy indicated, that nitrate reductase contained per M_r 172000 about 13 gatoms of iron (Table III). The content of acid-labile sulfur was slightly lower than that of non-heme iron. Chemical analysis of the enzyme suggested one gatom of Mo per 172000, even though the enzyme in its purified form had a tendency to be partially depleted of molybdenum.

The electronic spectrum of nitrate reductase is shown in Fig. 4. The enzyme is brownish and shows from a weak maximum at 410 nm a continuous decrease in absorbance towards 600 nm. The absorbance from 300 to 400 nm may be attributed to the iron-sulfur groups. Reduction of the enzyme by dithionite decreased the absorbance in the visible range. A small shoulder persisted at 420 nm. The ultraviolet spectrum had a peak at 280 nm and a shoulder at 290 nm. The ratio A_{280nm}/A_{410nm} of 4.7–6.3 is within the range of values determined for the *P. aeruginosa* [31] and *E. coli* [5] enzymes. None of our nitrate reductase preparations gave indications for the presence of cytochrome *b* or of flavin.

Release of molybdenum cofactor

The supernatant of thermally denatured nitrate reductase from *P. stutzeri* complemented the *nit-1* extract of *Neurospora crassa*, which lacks the molybdenum cofactor [23], and resulted in NADPH nitrate reductase activity (Table IV). Molybdate in the complementation mixture was essential; without added molybdenum during boiling, cofactor activity was only about half. The appearance of cofactor activity resulted in the loss of methyl viologen-dependent respiratory nitrate reductase activity. Cofactor activity was not detected in controls from which the enzyme or *nit-1* extract were omitted. The molybdenum cofactor of *P. stutzeri* nitrate reductase is a molybdopterin derivative of about 710 Da [34]. Recent evidence from the molybdoenzyme dimethyl sulfoxide reductase indicates that guanine dinucleotide is part of the cofactor, lending a higher mass to the bacterial molybdopterin [36]. It remains to be seen whether this nucleotide is part also of the molybdenum cofactor of respiratory nitrate reductase.

TABLE IV

Evidence for molybdenum cofactor activity of P. stutzeri respiratory nitrate reductase

Purified nitrate reductase (spec. act. 29 U/mg protein) was denatured at 100°C for 30 s in the presence or absence of 20 mM Na_2MoO_4. After cooling on ice and centrifugation, the resulting supernatants were used for the complementation assays with extracts from strain *nit-1* of *N. crassa*. Complementation was done at 30°C for 20 min in the presence of 20 mM molybdate.

Treatment of enzyme	Nitrate reductase activity (mU)	Cofactor activity (mU)
None	4900	54
Boiled, without Mo	0	339
Boiled, with Mo	10	716

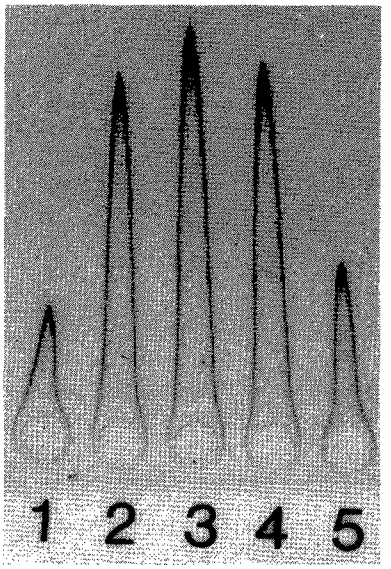

Fig. 5. Overproduction of respiratory nitrate reductase in *P. stutzeri* by azide. Extracts of cells grown in 12 mM nitrate-containing ACN medium were assayed by Laurell immunoelectrophoresis. Each lane was charged with 3 μl of crude extract (17 μg of protein) or enzyme. Lane 1, cells grown without azide; lanes 2 to 4, extracts of cells grown with 0.05, 0.1, or 0.2 mM of azide, respectively. Lane 5, 0.375 μg of purified nitrate reductase. The specific nitrate reductase activities of the samples were, for lanes 1–4, 0.3, 1.3, 1.3 and 1.4 U/mg, respectively.

Overproduction of nitrate reductase

The synthesis of denitrification enzymes in *P. stutzeri* depends on the nature of the respiratory substrate and a lowered oxygen tension in the culture medium. Maximum nitrate reductase levels are found in the presence of nitrate and an oxygen concentration below 2 mg per liter [37]. The simultaneous presence of low amounts of azide resulted in a further increase in activity in crude extracts. Azide concentrations up to 0.2 mM had no inhibitory effect on growth of *P. stutzeri*. A 4-fold increase of cell-free activity was caused by 0.05–0.1 mM azide. Higher concentrations had no further stimulatory effect. The effect was not seen with whole cells assayed with lactate. Azide alone induced nitrate reductase activity comparable to that of nitrate alone. The increased nitrate reductase activity was due to a concomitant increase in enzyme levels, as shown by rocket immunoelectrophoresis (Fig. 5). The immunochemically detectable amounts of the denitrification enzymes nitrite reductase and nitrous oxide reductase were not affected in extracts of cells grown with azide (data not shown). Azide-enhanced nitrate reductase activities have been described for *Proteus mirabilis* [38] and *S. aureus* [39],

but were not proved there immunochemically to be the result of an overproduction of the enzyme.

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